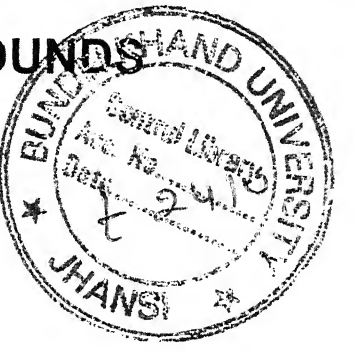
BIOTECHNOLOGY OF MASS PRODUCTION OF *Spirulina* WITH SPECIAL REFERENCE TO THE BIOLOGICAL COMPOUNDS



Thesis submitted for the degree

Doctor of Philosophy

in

Biotechnology

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By

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Dedicated

to

My Parents

CERTIFICATE

This is to certify that research work entitled "**Biotechnology of mass production of *Spirulina* with special reference to the biological compounds**" is a piece of work done by Ms. Radha Gupta under our guidance and supervision for the degree of Doctor of Philosophy in Biotechnology at J.C. Bose Institute of Life Sciences, Bundelkhand University, Jhansi, (U.P.) India.

To the best of our knowledge and belief the thesis:

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(3) Fulfils the requirements of the ordinance relating to the Ph.D. degree of the University; and is up to the standard both in respect of contents and language for being referred to the examiner.

Signature of the Supervisor

Signature of Co-supervisor

Signature of Head

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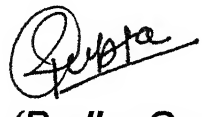

(Radha Gupta)

ABBREVIATIONS

APCI	atmoshpheric pressure chemical ionisation
ATP	adenosine tri-phosphate
BSTFA	bis-trimethyl silyl trifluoro acetamide
chl *a*	chlorophyll *a*
CLESS	controlled life and ecological support system
D	dark
DNA	deoxy ribonucleic acid
EDTA	ethylene diamine tetra acetic acid
FA	fatty acid
GC-MS	gas chromatography - mass spectrometry
g	gram
GLA	gamma linolenic acid
h	hectare
HEPES	4-(2-hydroxy ethyl)-1-piperazine ethane sulfonic acid.
h	hour
IP	ion pairing
Kb	kilobase
KD	kilodalton

Kg	kilogram
LC-MS	liquid chromatography-mass spectrometry.
L	light
l	litre
µg	microgram
ml	microlitre
µmol	micromol
ml	mililitre
M	molarity
mmol	milimol
min	minute
mg	milligram
MUFA	mono unsaturated fatty acids
N	normality
NiR	nitrite reductase
NR	nitrate reductase
NASA	national aeronautics and space administration
ODS	octadecyl silane
PDA	photodiode array.
PMSF	phenyl methyl sulphonyl fluoride

PS	photosystem
PUFAs	poly unsaturated fatty acids
SFA	saturated fatty acids
SDS-PAGE	Sodium dodecylsulphate-polyacylamide Gel electrophoresis
TBE	tris borate edta
TEMED	tetra ethyl methylene diamine
Tris	2-amino-2- (hydroxyl methyl) propane-1, 3diol
TMS	tri methylsilyl
UV	ultra-violet
V	volt
V/V	volume /volume
W/V	weight/volume
WHO	world health organization
ZB	zarrouk's basal
ZM	zarrouk's modified

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Introduction

1.1 Cyanobacteria

Cyanobacteria have unique biochemical status in the microbial world as oxygen evolving photosynthetic prokaryotes (Singh, 1950; Desikachary, 1959; Carr and Whitton, 1973; Stanier and Cohen, 1977; Rippka *et al.,* 1979; Carr and Whitton, 1982; Sprent and Sprent 1990; Singh *et al.,* 1995; Rai *et al.,* 1997; Adams, 2000; Whitton and Potts, 2000; Garcia *et al.*, 2000; Cavalier-smith, 2006; Gupta and Agrawal, 2006). The unique biological properties of cyanobacteria have enabled them to get exploited as fertilizer, nutrition and as biomedical value supplement (Banerjee *et al.,* 1996; Richer *et al.,* 1999; Zang *et al.*, 2001; Mishra and Pabbi, 2004; Aziz *et al.,* 2004; Khan *et al.,* 2005; Singh *et al.,* 2005; Rasool *et al.*, 2006).

Spirulina, a blue-green cyanobacterium, has existed on the Earth surface for more than 3.6 billion years (Henrikson, 1989; Skulacher, 1994). *Spirulina platensis* is a filamentous, photosynthetic, multicellular cyanobacterium belonging to the Oscillatoriaceae family, characterized by spiral shaped and enclosed in a thin sheath (Cifferi, 1983; Tomaselli, 1997). It is found in marine, brackish water and saline lake environments of

tropical and semi-tropical regions (Busson, 1971; Castenholz *et al.,* 2001).

Spirulina is most commonly used species of cyanobacteria for nutritional supplements (Kratz and Mayers, 1995; Blinkova *et al.,* 2001; Mazo *et al.,* 2004; Khan *et al.,* 2005; Singh *et al.,* 2005). It has been consumed by millions of people of all ages in the U.S. and 40 more countries for over 15 years (Furst, 1978; Cifferi, 1983; Kay, 1991; Belay *et al.,* 1994, 1996, 1997). Cifferi (1983), in his excellent review, explained the use of *Spirulina* by athletes from Mexico for a period 30-45 days, with good results in trials taken on Mexican children suffering from sever malnutrition, a significant weight gain was noticed. It is sold in tablets, capsules and powder. The dark green powder can be blended into fruit and milk-drinks or added to recipes to boost nutritional value.

Spirulina is rich in ingredients like essential amino acid (Richmond, 1992; Campanella *et al.,* 2002; Mazo *et al.,* 2004), vitamins (Switzer, 1980), essential fatty acids (Hudson and Karis, 1974;James and Carter, 1988; Huang and Mills, 1996; Cohen *et al.,* 1987; Mahazen and Kamat, 1995; Otles and Pire, 2001; Colla *et al.,* 2004), high protein content (Richmond, 1992; Campanella *et*

al., 2002), β carotene and phycocyanin that have nutritional and biomedical values (Becker *et al.,* 1986; Zang *et al.*, 2001; Mani *et al.*, 2002; Gemma *et al.*, 2002; Khan *et al.,* 2005). β carotene and phycocyanin contain anticancer activity (Peto *et al.*, 1981). *Spirulina* species contain very rich unusual nutritional profile (Blinkova *et al.,* 2001; Singh *et al.,* 2005). In view of the appearance of new viruses, drug-resistant bacteria, and ineffective antibiotics, scientists are searching the possibility of certain foods and food additives like *Spirulina* to boost the immune system and confront problems associated with early aging (Blinkova *et al.,* 2001; Khan *et al.,* 2005). This microorganism also finds application in bioremediation technology (Lodi *et al.,* 2003; Converti *et al.,* 2006).

Spirulina has applications in health foods, therapeutics and specialized feed (Ciferri and Tiboni, 1985; Zang *et al.*, 2001; Hirahashi *et al.*, 2002; Samules, 2002; Khan *et al.*, 2005; Singh *et al.*, 2005). Its impressive protein content and rapid growth in entirely mineral environments have attracted the attention of both researchers and industrialist alike. Keeping the aforesaid important facts in view, *Spirulina* has gained international

demands for its high value added biochemical constituents. The WHO (1992) has described *Spirulina* as one of the greatest super food on the Earth. NASA considers it an excellent compact food for space travel, as a small amount can provide a wide range of nutrients and it has been used in CLESS (Controlled Life and Ecological Support System) on a space station project NASA (Nishi *et al.,* 1987; Oguchi *et al.,* 1989). General crops are harvested once or twice a year. However, *Spirulina* can be harvested at an interval of 5 to 20 days (Switzer, 1980). It is possible to harvest *Spirulina* 50 times or more a year under suitable conditions. Therefore, *Spirulina* is expected as one means for solving the food problem of the Earth (Ciferri, 1983; Belay *et al.,* 1994, 1996, 1997; Mazo *et al.,* 2004).

As a result, *Spirulina* production may be carried out on a large scale in closed and open systems (Borowitzka, 1998; Costa *et al.,* 2003) for products like β carotene, phycocyanin (Bhasker *et al.,* 2005) and γ-linolenic acid (Lapinskas, 1999; Narayan *et al.,* 2005) production. Materassi, *et al.,* (1980), Torzillo and Carlozzi, (1996) and Watanabe and Hall, (1996) suggested first laboratory photo bioreactors for the production of *Spirulina* on a small scale.

Current world production of *S. platensis* for human consumption is more than one thousands metric tons annually. The USA leads world production followed by Thailand, India, Japan and China. Nutrient content depends on the location and environment in which the cyanobacterium grows (Clément, 1975). Percentage of specific components of *Spirulina* can be increased or decreased according to need by growing under regulated growth conditions.

1.2 *Spirulina* productions and processing technology

Spirulina grows naturally in the alkaline water of lakes in warm regions (Busson, 1971; Gupta and Changwal, 1988; Castenholz *et al.,* 2001). Costa *et al.* (2004) improved *S. platensis* biomass yield using a feed batch process. The harvesting techniques of *Spirulina* vary with climatic condition and geographical location. *S. platensis* is being harvested by cellular flotation and this method is also applicable to other cyanobacteria (Kim *et al.,* 2005).

Three kinds of commercial farms are operated today for mass production of *Spirulina*. In Ist case lake farms grow and harvest *Spirulina* from natural lakes. In IInd case outdoor pond

cultivation system could use open ponds or covered greenhouses for mass production of *Spirulina* (Henrikson, 1989; Costa *et al.,* 2003). In IIIrd case newly developed enclosed systems use transparent tubes or photo bioreactors (Pulz and Scheibenbogen, 1998; Khaled and Safia, 2003) for the mass production of *Spirulina*. Intensive approach consists of cultivation of pure strains of cyanobacteria in photobioreactors, which is more desirable than extensive ones (Pulz and Scheibenbogen, 1998; Khaled and Safia, 2003).

Generally, *Spirulina* is produced in open ponds through liquid cultivation system (Henrikson, 1989; Costa *et al.,* 2003). During liquid cultivation system the optical density of the culture medium is used to estimate the cell growth (Henrikson, 1989). Production of *Spirulina* in solid-state cultivation systems has been studied but in solid cultivation system estimation of cell growth is made difficult by the problems of separating cells from the cultivated medium (Senecal *et al.,* 1992; Cozza *et al.,* 1999; Pelizer *et al.,* 1999, 2000, 2002). As a result of these difficulties, biomass levels in solid-state fermentation systems are typically determined indirectly through the measurements of cell

constituents (Hesseltine, 1972; Huang *et al.*, 1978; Abdullah *et al.*, 1985; Gutierrez-Rojas *et al.*, 1995). Desgranges (1991a, 1991b,) compared four methods for biomass estimation during growth of *Beauveria bassiana* in solid-state fermentation systems. Some lakes such as Mexico, Myanmar, Chad and Lonar are well known to mass production of *Spirulina.* In India, research began in late 1970s, from backyard family scale to production farms (Becker, 1993).

On the basis of utility, *Spirulina* can be cultured under variable natural, artificial and laboratory conditions (Monteiro and Sunao, 2004). Zarrouk medium is frequently used during the isolation process (Zarrouk, 1966; Borowitzka, 1992), which is simple and highly alkaline ($NaHCO_3$) with pH range between 9 to 11. Like other microorganism, *Spirulina* has high specific growth rate ($0.3d^{-1}$) under optimal laboratory conditions, $0.2d^{-1}$ in natural condition during summer and $0.1d^{-1}$ in winter. Beside this, it has a very short life cycle ranging from 1 day under optimal laboratory condition and 3 to 5 days under natural conditions (Claudio, 1982).

1.3 Morphology

Spirulina is a multicellular, filamentous cyanobacterium. On microscopic observation it appears as blue-green filaments composed of cylindrical cells arranged in unbranched trichomes characterized by helical shape. The capsule has fibrillar structure and covers each filament protecting it. The irregular presence of capsule around the filaments in *S. platensis* is a differentiating morphological characteristic to compare with *S. maxima* (Balloni *et al.,* 1980; Belay, 1997). Trichome width varies from 6 to 12 µm, and is composed of cylindrical cells and helix diameter varying from 30 to 70 µm (Marty and Busson, 1970; Tomaselli, 1997). Although under certain conditions trichomes of *S. platensis* is as long as 20 mm (Van Eykelenburg, 1979).

The cell wall of *Spirulina* is composed of four layers L-1 to L-IV (Van Ekelenburg, 1977). The total wall thickness is about 60 nm and all layer are 10-15 nm thick. The septum separating the cells consists of peptidoglycan, which is coherent with peptidoglycan layer in the cell wall (Stanier and Cohen, 1977). The ultra structure and morphology of *S. platensis* is significantly affected by

environmental conditions and nutritional factors (Ciferri, 1983). Temperature influences cell size and occurrence of different cell organelles. The sheath surrounding the filament becomes more pronounced with increasing temperature, while pitch and diameter of trichome helix decreases (Van Eykelenburg, 1979). Cyanophycin granules are abundant up to 17°C, at which temperature polyglucan granules abruptly replace them. An increase in light intensity causes an increase in the concentration of gas vesicles and trichome length and a decrease in phycobilisomes (Borowitzka and Borowitzka, 1988). Carboxysomes are found to be present only when *S. platensis* was grown at high light intensities and in media containing a high nitrate concentration (Van Eykelenburg, 1979).

Three distinct morphological variant were recognized by Bai and Shashadri (1980) in an isolate of *S. fusiformis* from Mudurai. They are (i) S-type variant with trichomes having more or less regular and distinct coils (ii) C-type variant with trichomes having distinct spindle shape with close coils (iii) H-type variant with trichomes forming a dumb bell shape having very close to tight coils. They observed very distinct forms, which are readily

transformed from one type to another. In an attempt to identify the environmental factor that influences this conversion, they found that high light intensity and high nutrient concentration affected the transformation from S-type to C-type whereas high light intensity and low nutrient concentration enhanced the transformation of H-type variant from C-type. This phenomenon of morphological transformation is recognized as polymorphism.

1.4 Nutritional importance and value added biochemical of *Spirulina*

Spirulina is a suitable matrix for biotechnological incorporation of new food trace element preparation because it contain significant amounts of valuable proteins, indispensable amino acids, vitamins, beta-carotene, mineral substances, essential fatty acids, polysaccharides, glycolipids and sulpholipids etc. (Clément *et al.*, 1967; Dillon and Phan, 1993; Blinkova *et al.,* 2001; Ble-Castillo *et al.*, 2001; Campanella *et al.,* 2002; Colla *et al.,* 2004; Kapoor and Mehta, 1993; Mahajan and Kamat, 1995; James and Carter, 1988; Huang and Mills, 1996; Otles and Pire, 2001; Watanabe *et al.,* 2002; Switzer, 1980; Pyufoulhoux, *et al.,*

2001; Mazo *et al.,* 2004; Singh *et al.,* 2005). *Spirulina* also contains high level of various vitamins B, and minerals including calcium, iron, magnesium, manganese, potassium and zinc (Switzer, 1980; Pyufoulhoux, *et al.,* 2001; Blinkova *et al.,* 2001; Gireesh *et al.,* 2004). It is a good source of polyunsaturated fatty acid gamma linolenic acid (GLA) (Colla *et al.,* 2004; Otles and Pire, 2001). It has a soft cell wall made up of complex sugars and proteins (Balloni *et al.*, 1080). They are accepted as functional food, which are defined as products derived from natural sources, whose consumption is likely to benefit human health and enhance immunity (Hyashi *et al.,* 1994; Pugh *et al.*, 2001; Al-batsham *et al.,* 2001; Luescher-mattli, 2003; Lee and Werth, 2004). The addition of *Spirulina* to the diet can give a wide range of vital nutrients (Anusuya and Venkataraman, 1993; Belay *et al.,* 1993). 10 grams of *Spirulina* contains over 100mg of GLA (Nichols and Wood, 1986; Roughhan, 1989).

It contains ten times more protein than soya bean and three times to that of beef protein. It provides full compliment of nine essential amino acids. Dried *Spirulina* powder contains 65 to 70% protein (Ross and Dominy, 1990; Dillon *et al.,* 1995). *Spirulina* is

known to contain high percentage of glycolipids and sulpholipids (Kataoka and Misaki, 1983). It contains 5-8% lipid, from which 40% are glycolipids and 2-5% are sulpholipids. Sulpholipid is of great therapeutic value (Gustafson *et al.*, 1989). *Spirulina* contains high amount of bioavailable vitamin B_{12} and this is particularly important for vegetarians who often find it hard to get this nutrient in their diet (Harriman *et al.*, 1989; Leitzmann, 1993; Hau, 1995; Dagnelie *et al.,* 1991; Watanabe *et al.,* 2002). The Vitamin E levels of dried *Spirulina* are also high (Mitchell *et al.*, 1990; Gomez-coronado *et al.,* 2004).

Pigment content including chlorophyll and beta-carotene is also high. These pigments are called phycobilins, include phycocyanin and allophycocyanin (Miranda *et al.,* 1998; Hu and Liu, 2001; Gireesh *et al.,* 2004). Phycobilins are similar in structure to bile pigments such as bilirubin. In *Spirulina* cell, phycobilins are attached to proteins; the phycobilin-protein complex is called phycobiliprotein (Bhat and Madyastha, 2001; Pinero *et al.,* 2001). Studies have shown that the nutrients of *Spirulina* are readily absorbed by the body and help to bring nutrient status up to normal level. This is spatially true for minerals such as zinc and

iron and vitamins (Johnson and Shubert, 1986). It is also beneficial for malnourished children (Bucaille, 1990; Seshadri, 1993).

1.5 Environmental stress

Cyanobacteria are cosmopolitan and possess a high potential of adaptation to diverse environmental stresses since the period of pre cambrian era. They are probably one of the most important groups of organisms of serious global ecological importance and respond to the change in their environment (Rai, 1997; Nichols, 2000; Bhaya *et al.,* 2000; Gupta and Agrawal, 2006) among the behavioral response of aquatic cyanobacteria. Cyanobacteria are also important in many terrestrial environments and play a key role in maintaining the stability of the surface crust of semi desert and the fertility of soils used for farming in arid regions. Cyanobacteria are found in virtually all terrestrial niches and have been reported from locations which exhibit widely fluctuating chemical and physical properties including nutrient availability, light wavelength, temperature and water activity (Grossman *et al.,* 1994; Cavalier-smith, 2006). The present work is focused on the modifications and adaptation of photoautotrophic

metabolism in the filamentous cyanobacterium *S. platensis* in response to UV-B radiation.

1.5.1 UV radiation

UV-B radiation has been a ubiquitous problem for life and particularly for the photosynthetic organisms (Stapleton, 1992; Hader, 2000; Sinha *et al.,* 2001). All photoautotrophs, including the members of cyanobacteria depend on solar radiation as the primary source of the energy in their natural environment (Hader, 2000; Sinha *et al.,* 2001). The potential threat to these cyanobacterial communities is the continuous solar ultraviolet-B (UV-B 280-315) radiation reaching the Earth's surface due to depletion of the stratospheric ozone layer (Blumthaler *et al.,* 1990; Crutzen, 1992; Kerr *et al.,* 1993; Lubin *et al.,* 1995; Hader, 2000; Rezanka *et al.,* 2004). Recent studies show that the ultraviolet B (280–315 nm) (UV-B) irradiance reaching the Earth's surface has been increasing because of the depletion of the ozone layer (Kerr and McElroy, 1993; Lubin and Jensen, 1995; Hader, 2000; McKenzie *et al.,* 2003; Rezanka *et al.,* 2004), and this has attracted scientific attention to UV-B–related damage to the biota (Stapleton, 1992).

The increased UV-B radiation is detrimental to all forms of life, especially photosynthetic organisms that cannot avoid UV-B damage because of their requirement for light. Direct damage to key proteins, enzymes and DNA by UV-B and indirect oxidative damage to biomolecules are involved in the decrease in photosynthetic quantum yield, growth and survival (Quesada, and Vincent, 1997; Franklin and Forster, 1997). In particular, the leakage of electrons from the photosynthetic electron transport chain to oxygen and the photodynamic reactions of pigments such as chlorophylls, phycobiliproteins and quinines (Franklin and Forster, 1997) enhance the formation of reactive oxygen species (ROS) and tend to exert oxidative stress and oxidative damage to the organisms (Abeliovich and Shilo, 1972; Häder *et al.,* 1998; Mackerness *et al.,* 1998; An *et al.,* 2000; Vega and Pizarro, 2000).

UV-B radiation induces deleterious effects in all living organisms from prokaryotic bacteria and unicellular aquatic organisms to higher plants and animals (Stapleton, 1992; Hessen *et al.*, 1997; Hader, 2000; Sinha *et al.,* 2001; Rinalducci *et al.*, 2006; Holzinger and Lutz, 2006). Depending on the species, growth and survival decrease within a few hours of UV-B

irradiation. Biological effect of UV-B radiation includes DNA damage in most organism (Harm, 1980; Karentz *et al.,* 1991, 1991b), killing of bacteria (Kumar *et al.,* 2004), inhibition of motility (Donkor and Hader, 1995), and orientation, protein destruction, pigment bleaching and photoinhibition of photosynthesis in cyanobacteria (Krause, 1988; Cullen *et al.*, 1992; Hader *et al.*, 1998; Hader, 2000; Han *et al.*, 2001; Sinha and Häder, 2002; Sinha *et al*, 2003; Rinalducci *et al.*, 2006), and so on. Photodynamic reactions are potential mechanisms by which ultraviolet radiation induces damage to living cells (Ito, 1983; Sinha and Hader, 1996). UV-B effects on these organisms could also be relevant on a global scale.

For studying the effects of UV-B radiation on photosynthetic organisms, cyanobacteria are very useful model organisms because:

1) Cyanobacteria were among the earliest oxygenic photosynthetic organisms that were exposed in the course of their evolution to UV-B fluxes much higher than those presently reaching the Earth's surface.

2) They have developed several strategies for protection against the detrimental effects of UV-B.

Several studies have shown that photosystem II (PSII) is often sensitive to UV-B and it has often been assumed to be the most sensitive photosynthetic target for UV-B (Bornman, 1989; Melis *et al.,* 1992). However, UV-B-induced reductions in CO_2 assimilation can occur prior to, or in the absence of, depressions in PSII function and may more likely involve impairments in the Calvin cycle, possibly mediated by Rubisco (Nogués and Baker, 1995; Lesser and Neale, 1996; Allen *et al.,* 1999).

UV-B radiation leads to photoinhibition of photosynthesis thereby limiting the efficient fixation of light energy (Han *et al.* 2001, Nishiyama *et al.*, 2001; Rinalducci *et al.*, 2006; Bhandari and Sharma, 2006). Photoinhibition occurs due to two basic mechanisms: (i) photoinduced, nonphotochemical quenching of excitation energy and (ii) photoinduced damage to the photosynthetic machinery (Krause, 1988). In cyanobacterial photosynthesis, the nonphotochemical quenching particularly measured by O_2 evolution is not induced by light, indicating that

the photoinhibition is mainly due to the photoinduced damage to the photosynthetic machinery. The molecular mechanism of photoinhibition revealed that the light-induced damage is caused by inactivation of the D1 protein of the PSII complex (Aro *et al.*, 1993, Kanervo *et al.*, 1993). The damaged D1 protein is degraded proteolytically leaving the PSII complex depleted of the D1 protein. In the recovery process the precursor of the D1 protein is synthesized *de novo*, incorporated into the PSII complex, and then processed to yield the active D1 protein, with resultant generation of the active PSII complex (Andersson *et al.* 1992). The extent of the photoinhibition depends on the balance between the inactivation of the PSII complex and the recovery of the complex from the inactivated state (Gombos *et al.,* 1994).

1.6 Factors governing the production of *Spirulina* biomass

Successful cultivation of *Spirulina* is influenced by various physiological and environmental factors; better understanding of these factors is essential for further development of this biotechnology.

1.6.1 Carbon source

Since about 50% of the algal biomass consists of carbon a sufficient supply of carbon is of vital importance for successful cultivation. Carbon can be supplied as an inorganic substrate in the form of gaseous CO_2 or in the form of bicarbonate (Becker, 1994). High bicarbonate is required for mass cultivation of *Spirulina* in artificial systems. The amount of bicarbonate in Zarrouk's medium (Zarrouk, 1966), which is standard medium for *Spirulina*, is 16.8 g/l. In outdoor cultures supply of lower amounts of bicarbonate even at levels of 4.5 g/l resulted in yields comparable to that obtained with full amount of bicarbonate (Venkatraman and Becker, 1985). Like most cyanobacteria, *Spirulina* is an obligate photolithotroph and cannot grow in dark in media containing organic sources of carbon (Ogava and Terui, 1970). However, in light, it may utilize carbohydrates. The addition of 0.1% glucose to the growth medium enhances growth rate and cell yield (Ogava and Terui, 1972). Especially in dim light, mixotrophic growth results in cell yields that are two to three fold higher than corresponding yields obtained photoautotrophically (Ogava and Terui, 1970). *S. platensis* also have the ability to

utilize glycerol as the carbon source for growth (Narayan *et al.*, 2005).

1.6.2 Nitrogen and phosphorus

Adequate supply of nitrogen and phosphorus is imperative to ensure high production rates in mass cyanobacterial cultures. High yield coefficient, low crude protein content and low productivity were observed at reduced supply of these nutrients. The highest production rates were obtained at N and P concentration exceeding 25 and 2 mg/l respectively (Mosert and Grobbelaar, 1981). Since, *Spirulina* does not fix atmospheric nitrogen except for *S. labyrinthyformis*, a thermophilic species (Castenholz, 1976) an external nitrogen source is required for growth.

Generally, algae are able to utilize nitrate, ammonia or other organic sources of nitrogen such as urea (Becker, 1994; Costa *et al.*, 2001). Ammonium salts may be used as nitrogen source as long as NH_4^+ concentration is less than 100mg Nl^{-1}. Urea can be used as nitrogen source with no ill effects at pH 8.4 as long as its concentration is below 1.5% g/l (Borowitzka and Borowitzka, 1988). Zafaralla *et al.,* (1990) found that relative nutritive value was higher with sodium nitrate as source of nitrogen. Faintuch *et*

al., (1991) reported that maximum growth of *Spirulina* was obtained in presence of 2.57 g/l of potassium nitrate as compared to urea and ammonium nitrate. They reported that all nitrogen sources gave satisfactory growth rates. Manabe *et al.,* (1992) found that fatty acid production by *S. platensis* was influenced by ammonium chloride. *S. platensis* is reported to grow successfully in diluted human urine (used as a nitrogen source), and maximal biomass is obtained (Feng and Wu, 2004).

1.6.3 pH of medium

Spirulina is an alkalophilic organism by nature. Thus maintenance of high pH is mandatory in commercial production of *Spirulina*. Belkin and Boossiba (1971) while comparing the pH optima for cyanobacteria *Anabaena* and *Spirulina* demonstrated that optimal pH for *Anabaena* was in the range of 6.8 to 7.2 whereas the maximal growth rate for *Spirulina* was in 9.5-9.8 range (Rafiqul *et al.,* 2005; Ogbonda, *et al.*, 2006). When incubated at pH 7.0, the growth rate of *Spirulina* was severely inhibited. This high pH (>8) requirement clearly defines *Spirulina* as an obligatory alkaliphile (Grant *et al.,* 1990).

One of the major problems faced by cells in an alkaliphilic environment is that of regulating their internal pH. Belkin and Boussiba (1971) were the first to measure the ability of *Spirulina* to maintain a pH gradient across its cytoplasmic membrane with external pH values of 10.0 and 11.5, the intracellular pH values were only 8.0 and 8.5, respectively. Padan *et al.,* (1981) demonstrated that an active sodium-proton antiporter is required in order to maintain a low internal pH. Schlesinger *et al.,* (1996) demonstrated that *S. platensis* requires sodium in order to maintain optimal growth at pH 10. They demonstrated that under sodium deprivation the pH gradient collapses and the cells undergo fast lysis. High pH helps in two ways. Since the gas exchange between atmosphere and the culture medium depends on gradient of the partial pressure of gasses across the gas-liquid boundary layers, higher pH increases the CO_2 gradient under a given carbon alkalinity, which in turn enhances the contribution of free carbon from atmosphere. High pH also makes the culture medium exclusive for *Spirulina* and hostile raising the pH of the culture medium to 10.5 and further to 11.2 by the addition of sodium carbonate, is helpful to overcome the problem of

contamination with green unicellular algae like *Chlorella* and *Oocystis*.

1.6.5 Environmental factors

1.6.5.1 Temperature

The optimal temperature for laboratory cultivation of *Spirulina* ranges from 30-38^{0}C (Vonshak and Tomaselli, 2000; Ogbonda, *et al.*, 2006). However, many *Spirulina* strains differ in their optimal growth temperature as well as their sensitivity (Oliveira, *et al.*, 1999). Charenkova *et al.* (1975) reported that above 40^{0}C *Spirulina* cultures do not grow. Laboratory cultures kept at 45^{0}C for up to 24 hrs do not grow, but growth is resumed when the culture is brought back to 35^{0}C. They observed that above 45^{0}C, massive breakage of the trichome followed by cell lysis occurred. Even a brief period of exposure to temperatures around 50^{0}C, resulted in death of the cultures. The minimum temperature that permits some growth of *Spirulina* sp. is about 18^{0}C. During outdoor cultivation, when the maximum day temperature declines below 12^{0}C, the culture deteriorates. In contrast, *Spirulina* tolerate relatively low night temperature (Richmond *et al.*, 1980).

Tomaselli *et al.,* (1988) and Oliveira, *et al.,* (1999) observed that growth temperature also affects the biochemical composition of the cells. When *S. platensis* strain is grown under light-limited turbidostatic conditions at the maximum growth temperature (42^0C), a marked decrease in the photosynthesis pigments and proteins level was observed (Oliveira, *et al.,* 1999). Net biomass productivity of an algal culture is considered to be directly correlated to the net rate of CO_2 fixation and the rate of respiration. These two metabolic activities are highly dependent on temperature, while only the CO_2 fixation or oxygen evolutions are also light dependent. The optimum temperature for photosynthesis is 35^0C while dark respiration is highest at 45^0C (Torzillo and Vonshak, 1994).

1.6.5.2 Light

The availability of light to each cell in a photoautotrophic culture is a function of the intensity, duration of light irradiance, and the population density that affect the extent of mutual shading (Tamiya, 1957). In outdoor dense cultures, light is considered to be one of most important limiting factor (Vonshak and Guy, 1992). Dark respiration rate is affected by the light intensity at which cell

were grown. The higher the light intensity at which cells were grown, the higher the dark respiration rates. (Grobbelaar and Soeder, 1985; Torzillo *et al.,* 1991). These losses can be reduced by lowering the temperature of the culture. Algal strain with low dark respiration rate has advantage for mass cultivation (Vonshak and Richmond, 1988).

The phenomenon of photoinhibition has been studied extensively and well documented in algae and higher plants (Powels, 1984; Kyle and Ohad, 1986; Vonshak and Guy, 1992). Kalpan, (1981) observed a reduction in photosynthetic activity when the cells were exposed to strong light under carbon dioxide depleted conditions, and suggested that the reduction in photosynthetic activity was due to accumulation of hydrogen peroxide, a phenomenon known as photoinhibition. Olguin *et al.,* (2001) demonstrated that low light flux and nitrogen deficiency effet the chemical composition of *Spirulina* sp.

Different strains of *Spirulina* may differ in their sensitivity to photoinhibition. Vonshak *et al.,* (1988b) demonstrated that the difference in sensitivity in at least one strain was probably due to a difference in turn over specific protein, D1, which is a part of PS II.

The different responses of different *Spirulina* strains to photoinhibitory stress may be genotypic characteristics, as well being dependent on growth conditions (Vonshak and Tomaselli, 2000). Cultures grown at high light intensities exhibit a higher resistance to photoinhibition (Vonshak *et al.,* 1996). Photoinhibition is not only defined by the photon flux density but also the given temperature associated with it. The extent of photoinhibition was much higher when applied at temperature over or below the optimum for photosynthesis (Torzillo and Vonshak, 1994; Vonshak, 1997).

1.6.5.3 Oxygen

Oxidative stress in photosynthetic organisms is traditionally associated with excessive absorption of light energy by reaction centers. The greater photosynthetic activity under high light intensity is associated with exposure to increased oxygen levels, resulting in the inhibition of photosynthetic activity at first stage and leading to photoxidative death at later stage (Abeliovich and Shilo, 1972; Krause, 1994).

Vonshak *et al.,* (1996) reported that 32-hour exposure of laboratory cultures of *Spirulina* to pure oxygen resulted in

chlorophyll destruction due to photoxidative damage. This work indicates that photoinhibition and photooxidation phenomenon, although closely associated under similar environmental condition, may be parallel processes under stress conditions and should be considered as two types of stress with different sites of inhibition.

1.6.5.4 Evaporation

A major problem with algal cultivation in dry tropical areas is the high rate of evaporation from the open pond surface. Evaporation poses a problem both from the point of increasing salt concentration in the medium and in the acquisition of sufficient water to make up for the water loss (Becker, 1994). Measurements of the amounts of water evaporated during 24 hour period showed that during an-average summer day with a maximum air temperature of 37^0C and a water temperature of 30^0C approximately 10 litres water is lost from per m^2 of culture area. During monsoon, evaporation is less, but a minimum evaporation of 5 litres per m^2 is expected (Venkatraman and Becker, 1985).

In large open channel raceways in arid zones, 1-2 cm column of water day^{-1} can be lost (Vonshak, 1987) in 15-20 cm deep culture operated in continuous mode by recycling the culture

medium. This evaporation leads to a continuous increase in salt concentration in the medium. In two months periods the salinity may become twice the original level inducing osmotic stress. However, it has been demonstrated that *Spirulina* can be grown under elevated salt concentration without any significant reduction in photosynthetic oxygen evolution (Vonshak and Guy, 1985). A reduction in output rate can be expected owing to elevated activity of dark respiration. Vonshak *et al.,* (1996) proposed that a strain well adapted to increasing osmoticum without a significant increase in its respiratory activity would be advantageous for outdoor biomass production.

1.6.6 Cultural factors

1.6.6.1 Inoculum density

Optimum initial concentration of cyanobacterial cultures is important for economic cultivation of cyanobacteria. Very low initial concentration of the culture may lead to loss of culture due to photooxidation. Very high initial concentration on the other hand results in losses due to respiration and inefficient use of light energy due to self-shading (Venkatraman and Becker, 1985).

1.6.6.2 Culture depth

Vonshak *et al.,* (1982) reported that culture depth has a very significant effect on optimal population density i.e. the destiny, which yields the highest aerial output. They reported that reducing the culture depth by one half i.e. from 15.0 to 7.5 cm greatly increased the algal concentration at which maximal aerial productivity was obtained. Richmond and Grobbelaar, (1986) reported that the depth of the culture had no effect on maximum aerial output of dry biomass, but exerted a very marked effect on the optimal population destiny. Venkatraman and Mahadevaswamy, (1992) reported that pond depth of 18-20cm is optimum and any depth beyond this will merely add volume of the culture without correspond increment in yield of biomass.

1.6.6.3 Agitation

Sufficient agitation of the culture medium plays an important role in mass cultivation of cyanobacteria. Agitation involves a combination of various effects, viz., prevention of thermal stratification, avoidance of algal setting, homogenous distribution of nutrients, better utilization of carbondioxide, uniform dispersion of algae to ensure frequent exposure to light and avoid mutual

shading (Venkatraman and Becker, 1985). MCRC project at Madras, which is located near the coast of Indian ocean, steady flow of wind current was utilized through wind mill for agitating *Spirulina* cultures. Lawlor, (1974) developed a simple algal cultivation plant at Auroville, India, used a rotor constructed with plywood and canvas for stirring culture in a circular cement basin of 20,000 litres capacity. At CFTRI Mysore, three different methods were used for agitation of the culture i.e. (a) motor driven paddle wheals (b) pumping combined with gravity flow and (c) manual stirring. A turbulence of 25 to 30 cm/sec with the help of motor driven paddles is considered to be optimum for *Spirulina* (Venkatraman and Mahadevaswamy, 1992).

Venkatraman and Becker, (1985) described that *Spirulina* can be cultivated without continuous agitation. To achieve reasonable growth rate it is sufficient if the culture is stirred twice a day for about 15 minutes using broom bushes. The simple method does not require any energy inputs and is practical in the low level technology in India.

1.6.6.4 Outdoor cultivation systems for commercial production of *Spirulina*

Commercial large-scale culture of microalgae started in the early 1960s in Japan with the culture of *Chlorella,* followed by *Spirulina* in the early 1970s at Lake Texcoco, Mexico. The third major microalgae industry was established in Australia in 1986. The first plant in USA (Earthrise Farms) for the exploitation of *Spirulina* was built in 1981 in California, emerged as the result of a research work on its culture by Dainippon Ink and Chemicals, Inc. of Japan and Proteus Corporation of California (Vonshak, 1997). First small scale experimental plant using synthetic media was developed by the French Institute of Petroleum (Santillan, 1982). Since then different kinds of systems have been developed and operated at experimental, pilot and industrial scale. Currently two major systems are being used for commercial production of *Spirulina* (Venkatraman *et al.,* 1995). They are:

- Open cultivation systems
- Closed cultivation systems

1.6.6.4.1Open cultivation systems

The open systems have low production cost, easy handling and high production of biomass. So it is frequently chosen for industrial production (Borowitzka, 1998; Costa *et al.,* 2003), with three different basic designs. They are:

- Circular ponds where agitation is provided by rotating arm. Oblong forms (raceways) that are constructed either as single unit or in a joint form of several units (meander) with agitation by means of paddle wheels, propeller and air lift pumps.
- Sloping, often meander-like constructions where mixing of algal suspension is achieved by pumping and gravity flow (Becker, 1994).
- Open ponds are cheap units, easy to maintain. They permit through mixing of culture, avoid sedimentation and allow uniform exposure of the growing cells to light and nutrients.

Open ponds also have certain demerits. They are:

- The temperature required for *Spirulina* is not usually attained in open pond during winter season (Richmond *et al.,* 1980).

- Water column in the pond should be below 15 cm to reduce severe turbulence and respiration in flow (Venkatraman *et al.,* 1995).
- Low population density increases the production cost and cost of harvesting (Vonshak *et al.,* 1982).
- Contamination by other algae, bacteria, and zooplankton could often be a serious problem necessitating proper pond management (Venkatraman and Mahadevaswamy, 1992).

1.6.6.4.2 Closed cultivation systems

Closed photobioreactors were basically developed to maintain axenic cyanobacterial culture because contaminants can substantially reduce the quality and overall yield of the cyanobacterial product (Richmond *et al.,* 1993; Pulz and Scheibenbogen, 1998; Khaled and Safia, 2003). The major components of a bioreactor are:

- Photoreactor an illuminated chamber or tubular transparent structure generally made up of polyethylene or plexiglass material in which cyanobacterial growth occurs (Pulz and Scheibenbogen, 1998).
- A carbonation device for continuous CO_2 supply.

- A pump either for circulating the cyanobacterial culture within the system or out of the system for harvests. The pump may be substituted by an air compressor in case of airlift photobioreactor (Venkatraman *et al.,* 1995).

The demerits of a closed system are:

- It heats up quickly demanding for either a cooling device or selection of a thermotolerants strain.
- Production costs are very high accounting for over 50% of the cost of cyanobacteria (Tapie and Bernard, 1988).
- Cells stick to the inner wall of culture tube or tank; not only inhibiting light penetration but also remaining inaccessible for harvest, thereby adding decayed biomass to subsequent batches (Venkatraman *et al.,* 1995).

1.7 Yields and economics of production

The yield of cyanobacterial biomass is normally expressed as grams (dry matter) produced per square meter (culture area) or tones per hectare per year (Becker, 1994). There exists large variation of yields of *Spirulina* in commercial plants. At CFTRI, Mysore yields of 8-12 g/m^2/day were recorded (Venkatraman and Becker, 1985); whereas in Bangkok yields as high as 15 g/m2/day

were recorded. Algal production unit of Sosa texcoco, Mexico reports yield of 10 g/m^2/day. These yields are considerably lower than those estimated from laboratory experiments on small pilot plants, due to the influence of environment factors, during production under outdoor conditions.

In countries like United States where *Spirulina* is produced in clean water the cost of production is US $ 12/kg (Vonshak and Guy, 1992), whereas according to estimates by Venkatraman *et al.,* (1995), the cost of production of food grade material is less than Rs. 250/kg. Thus, there is ample scope of still lowering the cost of production by using cheaper inputs, which are easily available and increasing yields of *Spirulina* by optimizing growth parameters, to meet the needs of quality protein source for the increasing population.

1.8 Future scope

Cyanobacteria are probably one of the most important groups of organisms of serious global importance as they populate various different environments.

Molecular biology and genetics of *Spirulina* holds promise for new products and improved yield. At present, it is very difficult to say whether cyanobacteria would be in a position to compete *E. coli* or Yeast strains in heterotrophic expression of proteins. Many new ideal concepts are yet to be developed to exploit maximum benefit from cyanobacteria as presently being taken from *E. coli* and Yeast. The most important in this field is to think for production of secondary metabolites having role in pharmaceutical and processing, textile and cosmetic industries.

The extensive coastal belt of India and brackish lake Chilka provide an access to cultivation of various marine algae, especially cyanobacteria to compete world market in single cell proteins for its various secondary metabolites having extreme commercial value.

Spirulina has attained the status of a health food, because of its effect on beneficial intestinal flora, especially *Lactobacillus* and *Bifidus*. Maintaining a healthy population of these bacteria in the intestine reduces potential problems from opportunistic pathogens like *E. coli* and *Candida albicans*. When *Spirulina* is added to the

diet, beneficial intestinal flora is increased. *Spirulina* has a high content of nutritionally important compounds e.g. protein, amino acids, certain pigments, and polyunsaturated fatty acid (PUFA).

Improvement in the growth conditions including the growth media may lead to a significant enhancement in the productivity and the quality of the biomass. *Spirulina* is the only green food rich in rare polyunsaturated fatty acid gamma linolenic acid (GLA) with medicinal properties. Based on preliminary research, scientists hope that the use of *Spirulina* and its extract may reduce and prevent cancers and viral diseases. Bacterial or parasitic infections may be prevented or respond better to treatment and wound healing. Symptoms of anemia, poisoning and immunodeficiency may also be alleviated.

In the present *in vitro* studies, we attempted to investigate the effect of the UV-B radiation on the growth pattern, morphology and synthesis of biological compounds in *S. platensis*. It is for the first time that the investigations has been made for the changes in the biological compounds in *S. pletensis* under various stresses

including UV-B using modern sophisticated instruments viz. LCMS and GCMS.

1.9 Objectives

Following experiments were performed in order to meet the objective of the problems:

1. Selection of *Spirulina* strain and optimization of growth conditions for better yield of *Spirulina.*
2. To study effect of UV-B radiation on the growth and biochemical composition of *Spirulina platensis*
3. To study the effect of UV-B radiation on structure of *Spirulina platensis*
4. To study the effect of UV-B radiation on nitrate, nitrite uptake, NR and NiR activity of *Spirulina platensis*
5. Isolation, purification and characterization of thylakoid membrane of *Spirulina platensis* under UV-B stress
6. Characterization of chl *a* (from thylakoid membrane) through LC-MS in *Spirulina platensis* under UV-B stress

7. To study the changes in the expression of protein profile of *Spirulina platensis* under UV-B stress
8. To study variability of fatty acids and n-alkanes through GC-MS in *S. platensis* under UV-B stress

Materials and Methods

This chapter embodies the details of isolation and maintenance of experimental materials and methods employed through out the course of investigation.

2.1. Sterilization

The growth medium used for the routine culturing of *Spirulina platensis* was steam sterilized in an autoclave at a steam pressure of 15 lb per square inch at a temperature of 121°C. All the glasswares were sterilized in a hot air oven at 160°C for 2 hrs before being used. Some heat labile solutions were filter sterilized using membrane filter having the size 0.4 micron.

2.2 Organism

Pure culture of *Spirulina platensis* was procured from Division of Microbiology, Indian Agricultural Research Institute (IARI), New Dehli.

2.3 Maintenance of cyanobacterial culture

Spirulina platensis was axenically grown in Zarrouk"s medium (Zarrouk, 1966). Cultures were incubated in a culture room at temperature of 30± 2^0C and illuminated with day-light

fluorescent tubes having a photon fluence rate of 50 $\mu molm^{-2}$ s^{-2} at the surface of the vessels. Unless otherwise stated, all the experiments were carried out with log phase cultures having a cell density of 10μg chl a ml^{-1}. During the process of growth the flask was shaken 3 to 4 times per day. The experiments were run in duplicates. All manipulation involving the transfer of cultures in the liquid media or on agar plates were carried out under aseptic conditions on a laminar flow.

2.4 UV-B treatment

Exponentially growing cultures were harvested, resuspended in fresh Zarrouk medium and transferred to sterile Petri dishes (25 mm in diameter) for exposure to artificial UV-B radiation. The broad spectrum of UV-B (280-315) generated from a UV-B lamp (TL12 20 W fluorescent tubes, Philips, Holland), was used to irradiate the cells. The intensity of UV-B radiation falling upon the cells was measured by an international Light Radiometer/Photometer (type IL 1350, Japan) (Gour *et al.*, 1997).

Zarrouk's Medium

Table 2.1 : Composition of Basal Zarrouk's Medium

Ingredients	(g/l)
Sodium bicarbonate	18.00
Dipotassium hydrogen phosphate	0.50
Sodium Nitrate	2.50
Potassium Sulphate	1.00
Sodium Chloride	1.00
Magnesium Sulphate	0.20
Calcium Chloride	0.04
Ferrous Sulphate	0.01
Ethylene Diamine Tetra Acetate	0.08
A_5 Solution	1 ml

Table 2.1.1: Composition of A_5 solution stock

Ingredients	(g/l)
Boric acid	2.860
Magnese Chloride	1.810
Zinc Sulphate	0.222
Sodium Molybedate	0.0177
Copper Sulphate	0.079

Table 2.2: Composition of Modified Zarrouk's Medium

Ingredients	(g/l)
Sodium bicarbonate	16.8
Dipotassium hydrogen phosphate	0.50
Sodium Nitrate	2.50
Potassium Sulphate	1.00
Sodium Chloride	1.00
Magnesium Sulphate	0.20
Calcium Chloride	0.04
Ferrous Sulphate	0.01

Ethylene Diamine Tetra Acetate	0.08
A_5 Solution	1 ml
B_6 Solution	1 ml

Table 2.2.1: Composition of A_5 solution stock

Ingredients	**(g/l)**
Boric acid	2.860
Magnese Chloride	1.810
Zinc Sulphate	0.222
Sodium Molybedate	0.0177
Copper Sulphate	0.079

Table 2.2.2: Composition of B_6 solution stock

NH_4NO_3	0.023
$K_2Cr_2(SO_4)_2.24H_2O$	0.096
$NiSO_4.7H_2O$	0.048
$Na_2WO_4.2H_2O$	0.018
$Ti_2(SO_4)_3$	0.040
$Co(NO_3)_2.6H_2O$	0.044

2.5 Photomicroscopy

Photomicroscopy of UV-B untreated and UV-B treated cells of *S. platensis* were performed by using bright field microscopy, fluorescent microscopy and scanning electron microscopy (SEM).

2.5.1 Scanning electron microscopy

S. platensis cells were harvested and prefixed in culture medium by addition of an equal volume of 1% glutraldehyde in phosphate buffer. Cell were allowed to stand for 30 min on ice, pelleted, suspended in phosphate buffer with 2% glutraldehye and incubate for 1h at room temperature. Sample were washed with phosphate buffer and post fixed in 1% osmium tetraoxide in the same buffer and washed once in distilled water. Then sample were kept on carbon stubs and gold coating were done with fine coat ion sputter (JFC 1100). Sample observed with scanning electron microscope (JEOL JSM-840).

2.6.1 Measurement of growth

Growth was monitored by measuring increase in chlorophyll *a* (chl *a*) and protein content and was expressed in terms of

specific growth rate (μh^{-1}) and dry weight. The specific growth rate constant (μ) corresponds to ln $2/t_d$, where t_d is the doubling time. The specific growth rate (μh^{-1}) is computed following the method of Kratz and Myers (1955).

$$\mu = \frac{2.303 \times (\log N_2 - \log N_1)}{(T_2 - T_1)}$$

Where

N_1=Initial chlorophyll content

N_2=Final chlorophyll content

T_1=Initial time

T_2=Final time

2.6.2 Dry weight measurement

For dry weight measurement homogenous suspensions of known quantity of cyanobacterial sample were filtered through Whatmann No. 41 filter paper and oven dried at 75^0C till the filter paper attain constant weight.

The known volume of cyanobacterial sample were collected and filtered through pre weighed filter paper. After filtration, the filter paper was allowed to air dry and then transferred to a hot air oven at 75^0C for 4-6 hrs. The dried filter paper containing cyanobacterial biomass were cooled in vacuum desiccators and weighed. The difference between the initial and final weight were taken as the dry weight of cyanobacterial biomass. The dry weights were expressed in terms of g/l.

2.6.3 Chlorophyll *a* estimation

Chlorophyll *a* (chl *a*) was estimated following the method of Mackinney (1941).

Reagent

Absolute methanol

Procedure

A known volume (3ml) of cyanobacterial culture was harvested by centrifugation (5000 x g, 10 minutes) and the pellet were washed twice with distilled water (for *S. platensis* after five days of growth on centrifugation always not produce a pellet sometimes the cyanobacteria even float on the surface, in this

case 3 ml of homogenized cyanobacterial suspension were diluted with distilled water for overcome above mention problem).

The washed pellets were placed in 3 ml 100% methanol. The tubes were covered with aluminum foil and incubated at 4^0Cin refrigerator for 24 hrs for complete extraction of pigment. The extract were allowed to come at room temperature and centrifuged at 5000 rpm for 10 minutes. The absorbance of the methanolic supernatant was estimated at 663 nm using in Spectrophotometer (UV-visual Shimadzu, Japan) against methanol as a blank. Chl *a* ($\mu g\ ml^{-1}$) was quantified using the absorption coefficient of 12.63 as given by Mackinney (1941).

2.6.4 Measurement of protein

Cellular protein content was measured by the method of Lowry *et al.,* (1951).

Reagents

(a) NaOH 1 N

(b) (i) Na_2CO_3 5%

(ii) $CuSO_4.\ 5H_2O$ 0.5%

(iii) Na-K tartarate 1%

For making reagent (b) 50 ml of solution (i) was mixed with 1 ml of solution (ii) and solution (iii) each.

(c) Folin - ciocalteau reagent 1N

(d) Protein standard: Bovine Serum Albumin (BSA)

Procedure

To 0.5 ml of the cell suspension, 0.5 ml of the 1 N NaOH was added and mixture was kept in water bath for 10 min. After cooling, 2.5 ml of the reagent (b) was added and the mixture was further incubated at room temperature for another 10 min. This was followed by the addition of 0.5 ml of folin-phenol reagent (1N). After 15 min of incubation at room temperature, the color developed was read at 650 nm in a spectrophotometer (UV 1700, UV-Visible, Shimadzu, Japan) against a reagent blank. The concentration of protein in the sample was calculated from a calibration curve prepared by using bovine serum albumin (BSA fraction 5, Sigma, USA) as a standard.

2.6.5 Spectroscopic study

Absorption spectra of cyanobacterial suspension were measured from 400-700 nm with help of spectrophotometer (UV 1700 UV- Visible Shimadzu, Japan).

2.6.6 Turbidity

The growth rate of *S. platensis* was estimated by measuring the optical density of sample at wavelength of 650 nm. Known amounts of cyanoabcterial sample were centrifuged at 5000 rpm for 5 minute, washed once with distilled water. The cyanoabcterial filaments were suspended in 50% glycerol and absorbance read at 650 nm in a spectrophotometer (UV 1700, UV-Visible, Shimadzu, Japan) against a reagent blank (50% glycerol).

2.6.7 Carbohydrate estimation

The total carbohydrates were estimated by phenol-sulfuric acid method.

Reagent

(a) 5% Aqueous Solution of phenol

(b) 95.5% Sulfuric acid

(c) Carbohydrate standard: Glucose

Procedure

0.5 ml of homogenized cyanobacterial culture were taken in a tube and make a volume 2 ml by distilled water and 1 ml phenol solution were added. 5 ml sulfuric acid were added by rapid dispenser and mix thoroughly and allowed to stand for 10 minutes and placed in water bath for 15 minutes. After incubation period, the color developed was read at 492 nm in a spectrophotometer (UV 1700, UV-Visible, Shimadzu, Japan) against a reagent blank. The concentration of carbohydrate in the sample was calculated from a calibration curve prepared by using glucose (Sigma, USA) as a standard.

2.6.8 Fluorespectrophotometry

The fluorescence spectrum of the chl *a* of UV-B untreated and UV-B treated *S. platensis* were studied at an excitation wavelength of 435 nm using a fluorescent spectrophotometer (F-5000, Hitachi, Japan) having continuous light supply of tungsten lamp at room temperature.

2.6.9 Pigment analysis

2.6.9.1 Chlorophyll

Chlorophyll *a* was extracted in *Spirulina* strains with methanol and amount of total chlorophyll were calculated as described earlier.

2.6.9.2 Carotenoid

Carotenoid was extracted in acetone. Centrifuged the known volume of homogenous cyanobaterial suspension at 5,000 rpm for 10 min. washed the pellet twice with distilled water and homogenized the pellet with 3 ml 85% acetone. Stored the supernatant in the refrigerator for overnight. Centrifuged, and collected the supernatant. Repeat the extraction till acetone remains color less. Pooled the supernatant and make up to a known volume with acetone. Absorbance of crude extract was read at 450 nm using 85% acetone as a blank. Total carotenoids were calculated using the following formula

$$C = \frac{DxVxF}{2500}$$

Where,

C=Total Carotenoids in mg/ml

D=Absorbance at 450 nm

V= Volume of the extract

F=Dilution factor

2.6.9.3 Phycobiliproteins

Most commonly used method for the extraction of phycobiliproteins is repeated freezing and thawing of the sample in 0.05 M phosphate buffer. Centrifuged the known volume of homogenous cyanobaterial suspension at 5,000 rpm for 10 min. washed the pellet twice with distilled water suspend the pellet in 5 ml phosphate buffer and homogenized. Freezed thawed the content repeatedly and centrifuged at 5,000 rpm for 5 min. The quantity of phycocyanin (PC), C-phycoerythrin (PE) and allophycocyanin (APC) in mg/ml were calculated from the absorbance read at 562, 615 and 652 nm respectively, using the equation derived from the extinction coefficient of purified phycobiliproteins (Bennet and Bogorad, 1971).

$$PC = \frac{A_{615} - 0.474\,(A_{652})}{5.34}$$

$$APC = \frac{A_{652} - 0.208\,(A_{615})}{5.09}$$

$$PE = \frac{A_{652} - 2.41\,(PC) - 0.849(APC)}{9.62}$$

2.6.10 NO_3^-/NO_2^- uptake assay

Uptake of NO_3^-/ NO_2^- was assayed by measuring their depletion from the external medium (Bisen and Shanthy, 1991; Singh *et al.,* 1996). Exponentially growing untreated and UV-B treated cells of *S. platensis* were harvested by centrifugation, washed thoroughly with sterile Zarrouk'k medium, and resuspended in the same medium to a final density of 400 μg protein ml^{-1}. Assays were carried out in open flasks under aerobic conditions with continuous shaking at 25±1°C and at photon fluence rate of 50 μmol m^{-2} s^{-1}. Uptake was carried out by addition of 5 mM KNO_3/ KNO_2 to cell suspension at zero time. Samples were collected at regular time intervals and cell-free supernatants

were analyzed for residual NO_3^-/ NO_2^-. Linear portions of the curves were used to calculate uptake rate.

NO_3^- estimation

Procedure

To 1 ml of cell-free supernatant, three drops of brucine reagent was added and the mixture was shaken properly. 2 ml conc. H_2SO_4 and 2 ml distilled water were added. The mixture was incubated at room temperature in dark for 30 min and the absorbance of the color developed was recorded at 410 nm against a reagent blank (Manzano *et al.,* 1976). Concentration of NO_3^- present at different time interval was calculated with the help of calibration curve prepared by using KNO_3.

NO_2^- estimation

Procedure

To 1 ml of cell-free supernatant, 1 ml NED and 1 ml sulphanilamide were added and mixture was incubated at room temperature for 15 min. Absorbance of the color developed was recorded at 540 nm against a reagent blank (Snell and Snell, 1949). Concentration of NO_2^- present at different time interval was

calculated with the help of calibration curve prepared by using KNO_2.

2.6.11 NR/ NiR activity assay

In vivo, NR activity was assayed in toluene (2% v/v) permeabilized cells (Herrero *et al.,* 1981; Bisen and Shanthy, 1991). The permeabilized cells were incubated in 1 ml reaction mixture containing KNO_3/ KNO_2, 20 µmol; MV, an artificial electron donor, 4 µmol and $Na_2S_2O_4$ (a strong reducing agent used for reducing MV); 10 µmol in 0.1 ml of 0.3 M $NaHCO_3$ for 5 min at 30°C. NO_2^- formed was assayed by diazocoupling method (Snell and Snell, 1949) in corresponding media free from cells. Blanks were also prepared by stopping reaction at zero time.

2.7 Polyacrylamide gel elecrophoresis

The principle used in the polyacrylamide gel electrophoreisis (PAGE) is based on the fact that proteins are charged molecule which, when suspended in a medium and subjected to an electric field, migrate to the electrodes of opposite polarity. The formation of a polyacrylamide gel results from the polymerization of acrylamide monomer into long chains and the cross-linking of

these by compounds such as N'N'-methylene bisacrylamide. Thus, polyacrylamide gel can be considered as a porous medium through which proteins are separated according to either charge density or size.

The technique of PAGE using a discontinuous buffer system was introduced to improve the resolution of the separated proteins bands and the concentration of the dilute samples. PAGE was adopted for use with the detergent sodiumdodecyl sulphate (SDS) by Laemmli (1970). When denatured by heating in the presence of excess SDS and a thio reagent (e.g. mercaptoethanol), most proteins bind the detergent in a constant ratio of 1.4 g g^{-1} protein. Under this condition, the detergent introduces negative charges, which nullify the intrinsic charges of the protein. Thus, migration of polypeptides in SDS-PAGE largely depends on the size rather than the charge.

2.7.1 SDS-PAGE

In order to see the effect of UV-B radiation on protein profile of *S. platensis*, the cells were grown and the protein samples were prepared following the method of Borbely *et al.,* (1985).

2.7.1.1 Preparation of protein samples

Exponentially growing culture (100 ml) was harvested by centrifugation (5,000 rpm, 4°C) for 10 min. The pellet was washed, twice with distilled water. Pellets were suspended with lysis buffer (containing) incubated at 50°C for 45 minute. The resulting cell suspension was kept on ice, and the chilled suspension was disrupted by ultrasonication at amplitude of 25 microns for 10 min using an ultrasonic disintegrator (Soniprep, MSE, England) fitted with a titanium probe. The disrupted cell suspension was centrifuged at 1000xg for 10 min to remove the undisrupted cells. The supernatant was thoroughly mixed with 10% TCA and kept at 4^0C overnight. Precipitated proteins were centrifuged at 20,000xg for 20 min at 4^0C. The pellet thus obtained was resuspended in the sample buffer was stored at -20^0C. SDS-PAGE was carried out by using the gradient (12-25%) gel. Rest of the procedure was same as described by Laemmli (1970).

Reagents

(i) Sample buffer

The final concentrations of each of the ingredients in the

sample buffer were:

PMSF	1 mM
SDS	4% (w/v)
β-mercaptoethanol	8% (v/v)
Bromophenol blue	0.01% (w/v)
Glycerol	20% (v/v)

Final volume was made 10 ml by using Tris-HCl buffer (10 mM, pH 6.8).

(ii) Standard protein marker

Solutions containing 2 mg total protein in a mixture of the following markers proteins dissolved in sample buffer were used.

Proteins	**Molecular weight (kDa)**
Lysozyme	14.3
Trypsinogen	24.0
Ovalbumin	43.0
Bovine serum albumin	66.0
Fructose-6, phosphate isomerase	90.0
Phosphorylase	97.4

(iii) Acrylamide stock

Acrylamide	29.2% (W/V)
Bis-acrylamide	0.8% (W/V)

(iv) Running gel

Different concentrations of acrlamide gel were used for the preparation of gradient gel, which was obtained by varying the final concentration of acrylamide solution. The following amounts of other constituents were always added in the running gel (final volume 6 ml) except for acrylamide solution.

1.5M Tris-HCl (pH 8.8)	2.25ml
10% SDS	0.45ml
TEMED	0.0046
10% Ammonium per sulphate (Freshly prepared)	0.045

(v) Stacking gel (4%)

30% Acrylamide	2.5ml
Tris-HCl (0.5M, pH 6.8+0.4%SDS)	2.5ml

TEMED	10µl
10% Ammonium per sulphate	10µl
(Freshly prepared)	
Distilled Water	6.0ml

(vi) Running buffer

Tris	0.025M (pH 8.3)
Glycine	0.25M and
SDS	0.1%

(vii) Methanolic solution

Methanol	50 ml
Distilled water	50 ml

(viii) Staining solution

Coomassie blue-R250	0.5g
Isopropyl alcohol	25ml
Glacial acetic acid	10ml
Final volume (by distilled water)	100ml

(ix) Destainer

Glacial acetic Acid	25 ml
Double distilled water	225 ml

Procedure

Running gel was prepared by using the gradient concentrations (12 to 20%, w/v) stock solution of 30% (w/v) of acrylamide. The highest concentration (20%) of acrylamide was used for the first layer from bottom side while the 12% (w/v) of acrylamide gel solution occupied the top layer. After pouring the different concentrations of acrylamide, the top level was made smooth and straight by adding about 2 ml of 50% (v/v) methanolic solution. In this way all the concentrations of running gel were prepared. Each time methanolic layer was removed and washed with distilled water. The stacking gel was poured on the top layer of running gel and wells were made in the stacking gel by inserting the comb. After satisfactory polymerization of the gel, the combs were removed and the running buffer was used to fill the space in the sample compartment. Finally, the upper and lower buffer tanks were filled by the running buffer. The protein samples (60-100 µg) and the standard protein marker (ranging from 14.3 to

97.0 KD) were loaded simultaneously in the well. The solutions were heat-treated for 15 min at 100°C in boiling water bath prior to loading of the samples. The slab gel electrophoresis (Mini-protein(r) II Cell, Bio-Rad, USA) was carried out at 4°C using current of 10 mA with 100 V. The gel was removed when the tracking dye reached to its bottom and the bromophenol blue dye was not allowed to run out of the gel.

2.7.1.2 Staining and destaining

The gel was stained for 2 hrs in the staining solution prepared by using coomassie blue R-250 stock. The gel was destained by flooding it with destainer. After a regular interval of time, the destaining solution was replaced by the freshly prepared destaining solution. This process was repeated till the background of the gel become clear and protein bands were clearly visible with naked eye.

2.8 Isolation and purification of thylakoid membrane

The thylakoid membrane was isolated by sucrose density gradient with some modifications (Omata and Murata, 1984). Spheroplast was obtained by incubation of intact filaments in TES

buffer (0.4 M sucrose, 20mM TES, 10 mM NaCl, 10 mM EDTA), pH 8.0, containing lysozyme (0.5 mg/ml) overnight at 37°C. The spheroplasts were ruptured by French press cell at 0.7 Mpa in the presence of PMSF (0.1 μg ml^{-1}). Cell wall was removed by centrifugation at 18,000 x g for 30 min. Thylakoid and cytoplasmic membranes in the supernatant were concentrated by ultracentrifugation at 135,000 x g for 40 min, resuspended in TES buffer (20 mM TES buffer, 10mM NaCl), pH 8.0, then loaded on a 30-90% linear gradient of sucrose (w/v in TES buffer) and centrifuged at 110,000xg for 16 h. The separated bands were identified as

- A chlorophyll-less faint-yellow band of plasma membrane, at a sucrose concentration of 30-33% (w/v), containing 0.02-0.03mg protein/ml from a total of 0.2-0.3 mf protein ml-1 loaded on the sucrose gradient (Gabbay-Azaria *et al.,* 1992).
- Dark green band of thylakoid membrane, containing a major part of the protein loaded onto the sucrose gradient (0.7-0.09 mg ml^{-1}) at a sucrose concentration of 39-50% (w/v). Protein content was determination by the modified Lowry procedure. Chl *a* were determined according to Mackinney (1941).

2.8.1 LC-MS study of chlorophyll *a* of thylakoid membrane

Sample preparation of pigments

Samples for photopigment analysis were obtained by vacuum filtration on to a glass fiber filters (whatman GF/F). Filters was placed in microcentrifuge tubes 2ml, a known volume 0.5-1.5 ml of 100% acetone added in extract and sonicated for 30-60 sec in a ice slurry to reduce heating. Tubes are wrapped in aluminum foil, placed in freezer –20°C and extracted overnight 12 hr. After extraction the supernatant is filtered through 0.45μm PTFE filter. A known volume of the extract was then dispensed into a fresh vial. Just prior to run an ion pairing (IP) solution 1M ammonium formate is added to the vial in a ratio (3:1) of 3 parts extract and 1 part of ammonium formate.

Octadecyl silane (ODS) column, utilizing methanol/ propanol as mobile phase by using APCI (Atmospheric pressure chemical ionization) method for analysis of pigment chl *a* in untreated and UV-B treated *Spirulina*. Total ion chromatogram was taken and PDA detector was used for level of absorption of specific wavelength.

2.9 Extraction of fatty acids

Cells of *S. platensis* were harvested and fatty acids were extracted with haxane. Unsaponifiable fraction was discarded and the remaining aqueous phase was extracted with hexane and proceeds for fatty acid extraction (Qiu *et al.,* 2002).

2.9.1 GC-MS analysis of fatty acids

A Varian 2400 gas chromatograph with capillary column coupled to mass detector Finnigan mat TSQ (700) was used. On HP-5 column (30m, internal diameter 0.32mm, film thickness, 0.25mm). The GC oven was programmed as follows 5min at 45°C; 15^0C min^{-1} to 280^0C; and hold for 5 min at 280° C. The injector temperature was kept at 280^0C (splitless). The flow rate of carrier gas (helium) was 1.2ml $minute^{-1}$. The MS detector was operated at 150^0C, with electron impact ionization energy set at 70 eV. The scan range was m/z 40-650 and scan rate 0.9scans $second^{-1}$. Solvent delay was set at 4 min. Fatty acids was identified by comparison with standard fatty acids.

2.10 Extraction of hydrocarbons

Cells of *S. platensis* were harvested and hydrocarbons were extracted with haxane. Unsaponifiable fraction was taken for hydrocarbon analysis (Qiu *et al.,* 2002).

2.10.1 GC-MS analysis of hydrocarbons

A Varian 2400 gas chromatograph with capillary column coupled to mass detector Finnigan mat TSQ (700) was used. On HP-5 column (30m, internal diameter 0.32mm, film thickness, 0.25mm). The GC oven was programmed as follows 2min at 50°C; 15^0C min^{-1} to 250^0C; and hold for 5 min at 250° C. The injector temperature was kept at 250^0C (splitless). The flow rate of carrier gas was 2ml $minute^{-1}$. The MS detector was operated at 150^0C, with electron impact ionization energy set at 70 eV. The scan range was m/z 40-650 and scan rate 0.9 scans $second^{-1}$. Solvent delay was set at 6 min. Hydrocarbons was identified by comparison with those found in Wiley mass spectral library (7^{th} edition).

Results and Discussion

3.1 Growth study of *S. platensis*

Culture of *S. platensis* in conical flask has its limitation in providing complete information related to growth, development and production of value added chemicals viz. vitamins, amino acids, fatty acids, proteins and polysaccharides both in quantity and quality and disposing off carbon dioxide one of the major causes of global warming (Capone *et al.,* 1997; Ferreira *et al.,* 2004). Extensive research has been conducted on production of *S. platensis* (U.N. World Health Organization, 1993; Sassano *et al.,* 2004; Costa *et al.,* 2003, 2004) living at salt lakes in the tropical regions (Busson, 1971). The Chad Lake has ingested *S. platensis* as a protein source from time immemorial (Richmond, 1992; Dillon and Phan, 1993; Campanella *et al.,* 2002; Mazo *et al.,* 2004).

The protein of *S. platensis* is composed of lot of amino acids essential to human being (Richmond, 1992; Campanella *et al.,* 2002; Mazo *et al.,* 2004; Singh *et al.,* 2005), and richly contains minerals (Johnson and Shubert, 1986; Cohen *et al.*, 1997; Blinkova *et al.,* 2001; Pyufoulhouk *et al.,* 2001; Gireesh *et al.,* 2004) and nutrient substances except for vitamin C. *S. platensis* is

drawing public attention as a nutrition-supplementary food for human health (Mazo *et al.,* 2004). Therefore, *S. platensis* is expected as one means for solving the food problem of the Earth (Ciferri, 1983; Belay *et al.,* 1994, 1996, 1997; Mazo *et al.,* 2004; Singh *et al.,* 2005). WHO (1992) has described *S. platensis* as one of the greatest super food on the Earth and NASA considers it an excellent compact food for space travel, as small amount can provide a wide range of nutrients (Nishi *et al.,* 1987; Oguchi *et al.,* 1989; Khan *et al.,* 2005).

S. platensis has been usually cultured at various places in world under natural growth condition but the use of a culture pool, which has a relatively shallow depth for exposing the culture containing cyanobacteria to sunlight. *S. platensis* grows better in a liquid environment or culture medium of high pH and alkalinity (Busson, 1971; Grant *et al.,* 1990). It forms massive population in tropical and sub-tropical water bodies, characterized by high level of carbonate and bi-carbonate and high pH. The fame of *S. platensis* is a result of its economic importance, which is due to its nutritional and biomedical values (Khan *et al.,* 2005). The mass culture becomes attractive as a source of food feed and fine

chemicals (Richmond, 1992; Belay *et al.,* 1994, 1996, 1997; Singh *et al.,* 2005; Chamorro *et al.,* 2002).

Physico-chemical profiles of *S. platensis* is describing the relationship between growth and environmental factors especially irradiance flux, density and temperature (Vonshak and Tomaselli, 2000), which are important in the evolution of microalgae and cyanobacteria for biomass production, as well as their general characterization. High alkalinity is mandatory for the growth of *S. platensis* and bicarbonate is used to maintain high pH (Belkin and Boussiba, 1981; Grant *et al.,* 1990; Huang *et al.,* 2002). Source of nutrition also affect the growth rate of cyanobacteria (Faintuch *et al.*, 1991)

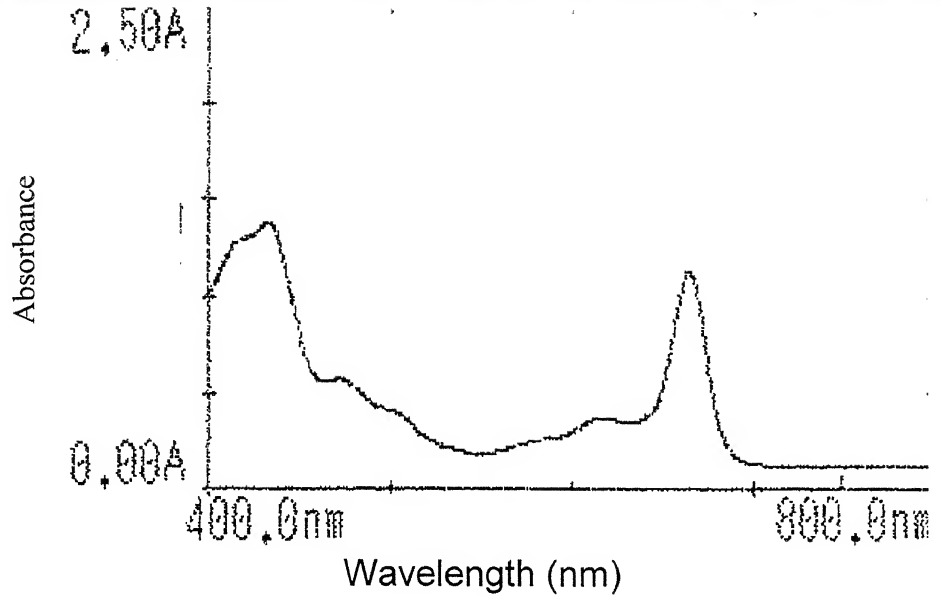
In the present study we investigated comparative growth rate of *S. platensis* on Zarrouks Basal (ZB) and Zarrouks Modified (ZM). The growth of *S. platensis* in flask culture was monitored and expressed in terms of dry weight, chl *a* content, specific growth rate, turbidity, protein content and protein profile when grown on Zarrouks Basal (ZB) and Zarrouks Modified (ZM) media.

Specific growth rate of *S. platensis* was 0.066 (μh^{-1}) on Zarrouk's basal medium and 0.052 (μh^{-1}) on Zarrouk's modified medium (Table 3.1). The data shows that specific growth rate of *S. platensis* is higher on ZM medium. The absorption spectrum of *S. platensis* on ZM culture medium was noticed to be more prominent as compared to ZB medium (Fig. 3.1 a, b). The absorption maxima of blue range (645, 665 nm) were having more optical density as compared to ZB medium. This shows that photo harvesting pigment was qualitatively increased on ZM culture medium.

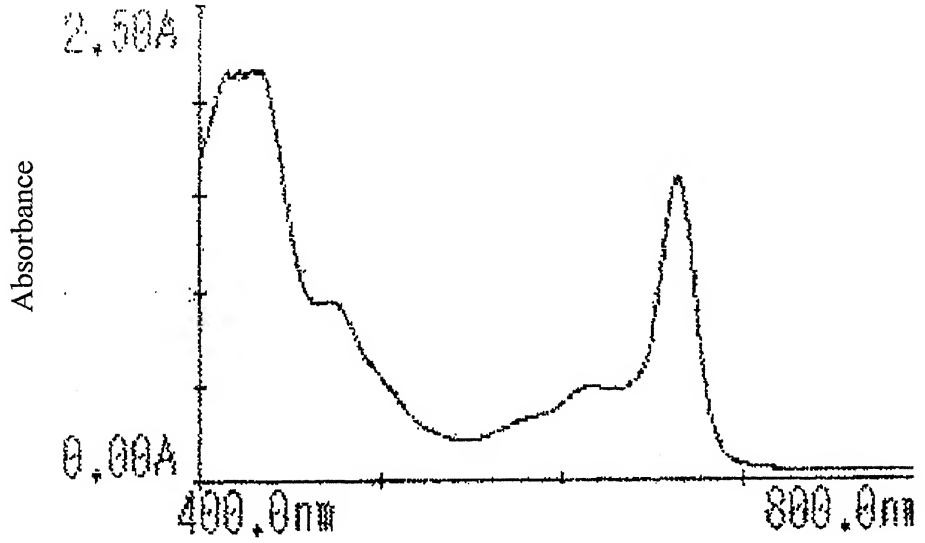
The chl *a* content of *S. platensis* was 24.805 μgml^{-1} on ZM medium and 19.387 μgml^{-1} on ZB medium (Fig. 3.2). The protein content of *S. platensis* on ZB medium was 610 μgml^{-1} and 733 μgml^{-1} on ZM medium (Fig. 3.3). It is evident from results that protein content of *S. platensis* is higher on ZM medium. Results on turbidity measurements revealed that the turbidity of *S. platensis* is higher on Zarrouk's modified medium as compared to Zarrouk's basal medium (Fig. 3.5).

Table 3.1: Effect of micro ingredient of Zarrouk's medium on growth: in terms of specific growth rate (μh^{-1}), dry weight (g l^{-1}) chlorophyll content ($\mu g ml^{-1}$) and protein content ($\mu g ml^{-1}$) of *S. platensis*.

S. No.	Growth parameters	Growth of *S. platensis* on Basal Zarruok medium	Growth of *S. platensis* on Modified Zarruok medium
1	Specific growth rate	0.052	0.066
2	Dry weight	1.15	1.25
3	Chlorophyll content	19.387	24.805
4	Protein content	610	733



(a)



(b)

Fig. 3.1 Absorption spectra of chlorophyll *a* of *Spirulina platensis* grown on Zarrouks Basal (a) and Zarrouks Modified medium (b)

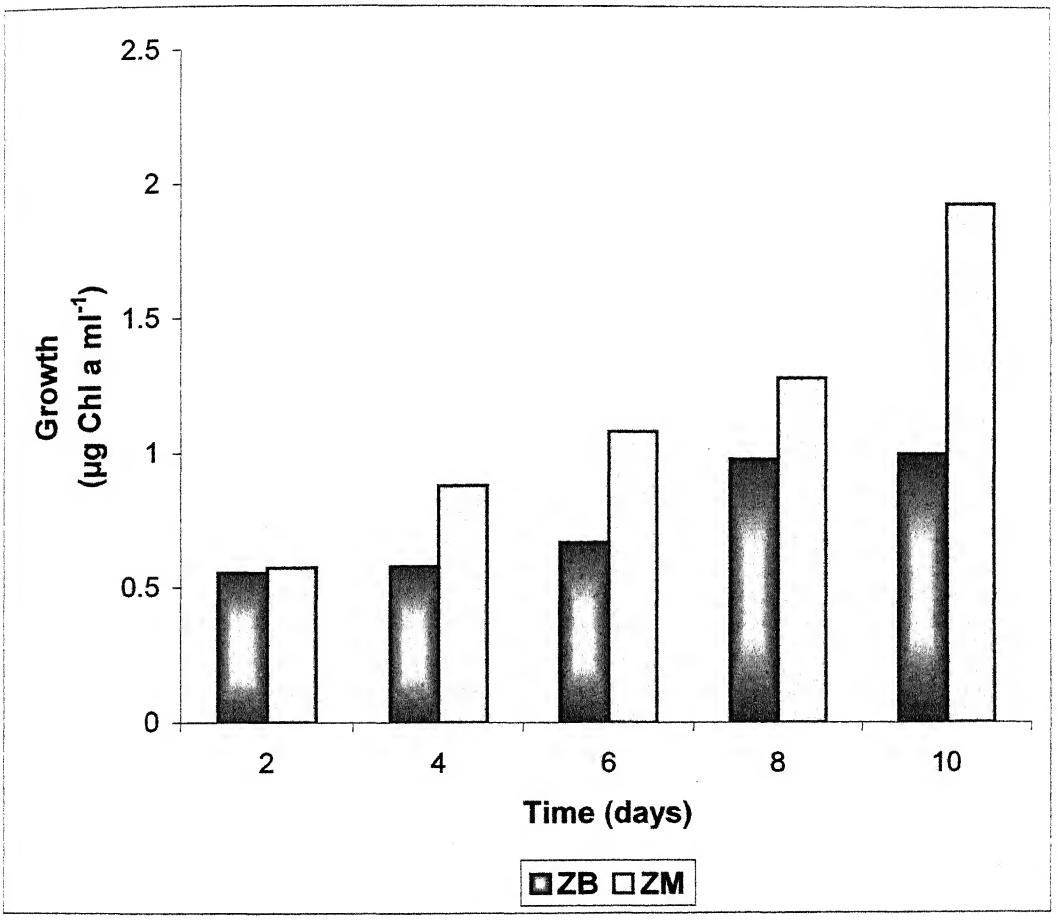


Fig. 3.2 Growth (in terms of chl *a*) of *Spirulina platensis* grown on Zarrouk's Basal and Zarrouk's Modified medium

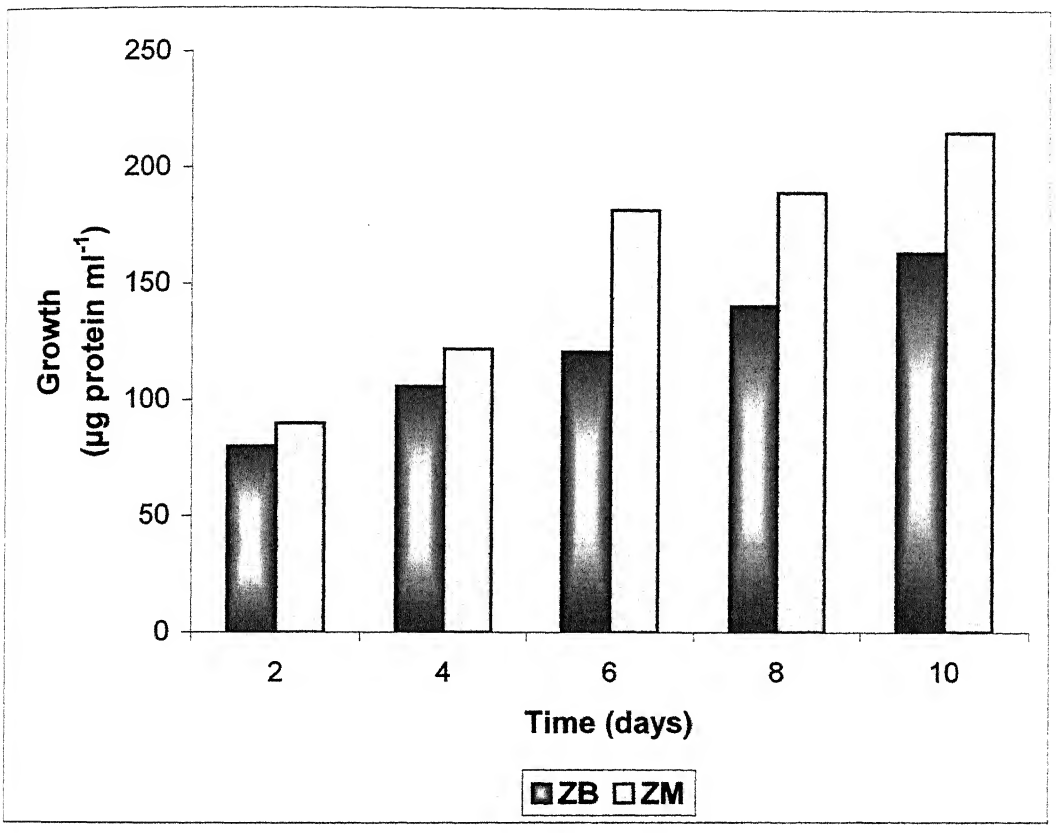


Fig. 3.3 Growth (in terms of protein) of *Spirulina platensis* grown on Zarrouk's Basal and Zarrouk's Modified medium

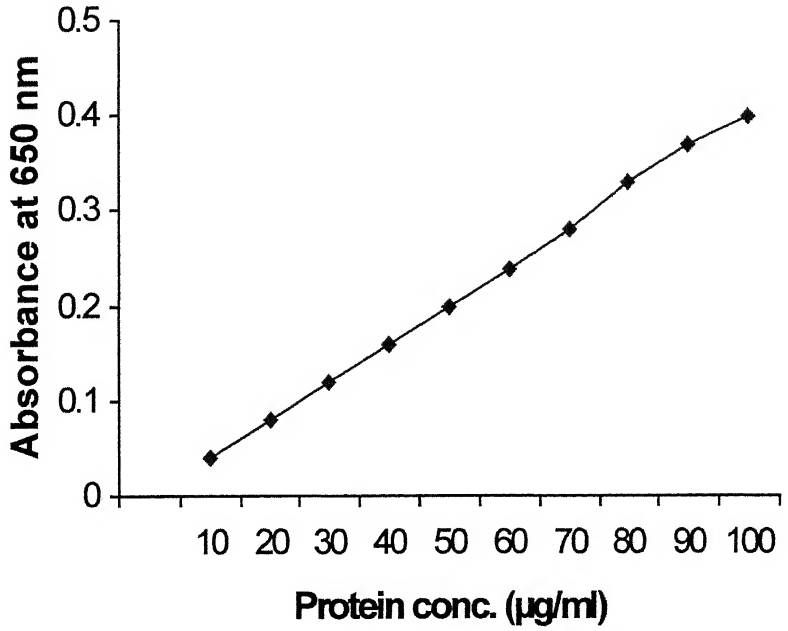


Fig. 3.4 Protein standard curve with BSA

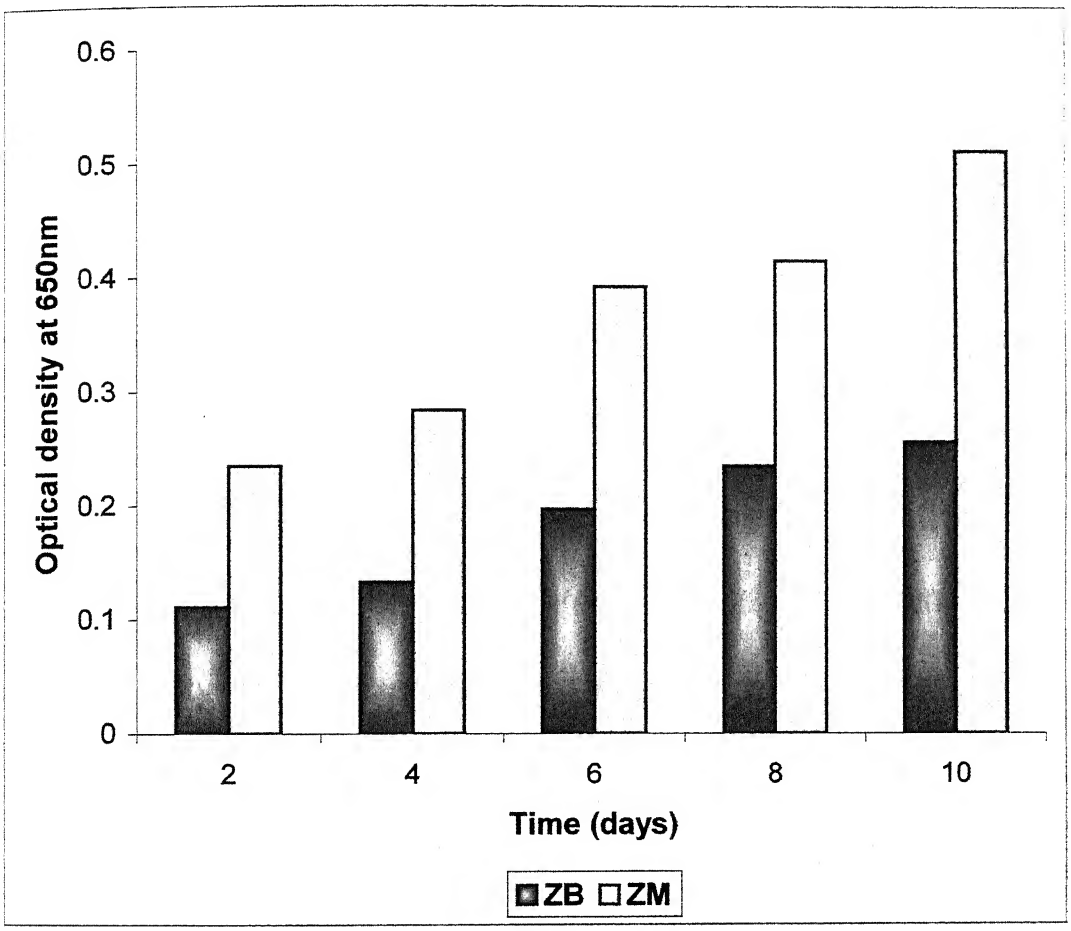
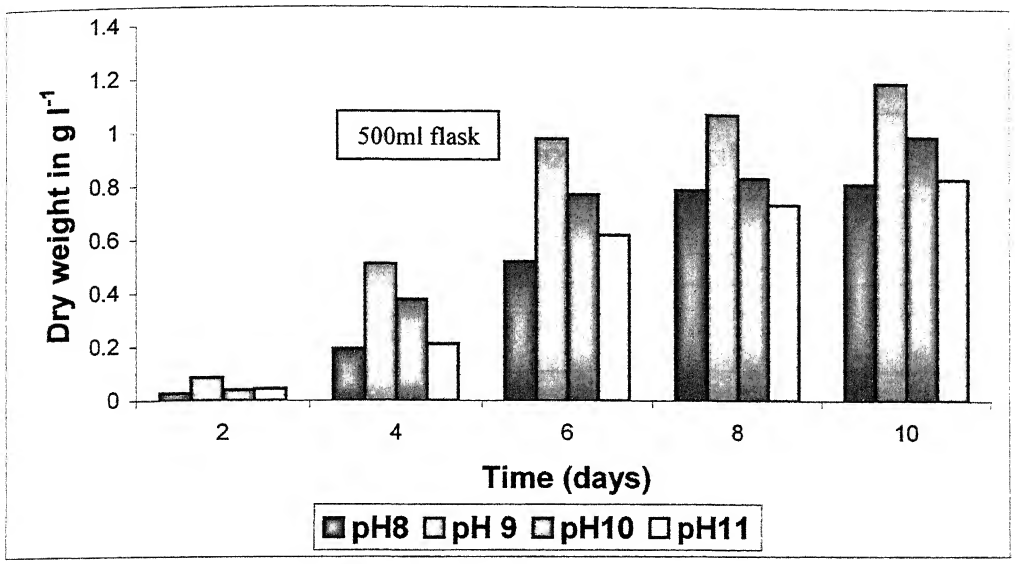


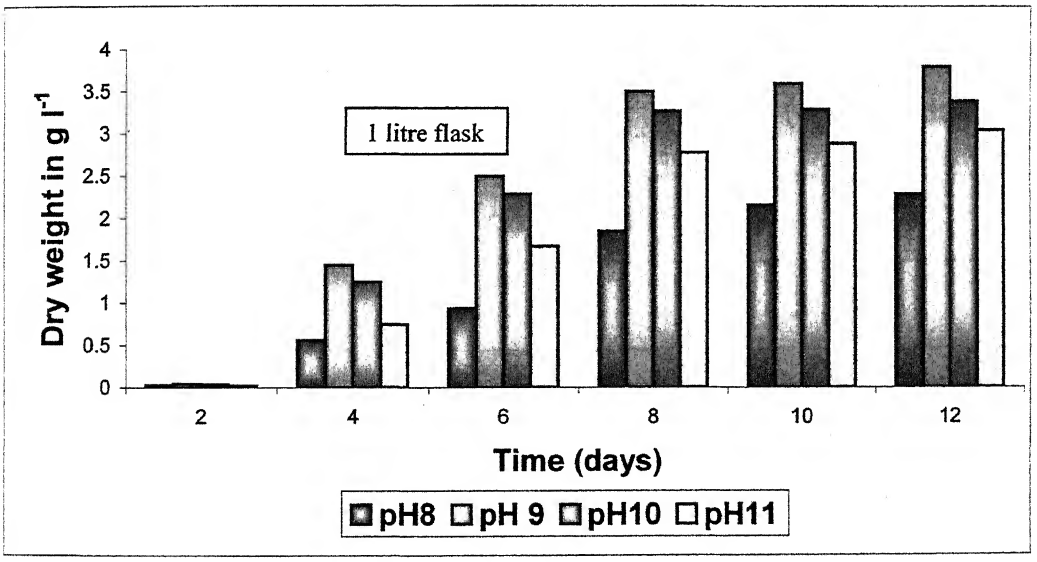
Fig. 3.5 Turbidity of *Spirulina platensis* on Zarrouk's Basal and Zarrouk's Modified medium

S. platensis was grown at different pH (8, 9, 10 and 11) in flask culture and monitored and expressed in term of dry weight (Fig.3.6a, b). The maximum bulk density about 1.25 g l^{-1} was noticed when the pH of culture medium was maintained at 9.0 with medium volume 250 ml in a 500ml flask. The maximum bulk density was attained on 10^{th} day of the culture with the increase of culture volume the maximum to one litre bulk density of *S. platensis* culture was shifted from 10^{th} day to 12^{th} day and remain to steady state for 24 hrs (Table 3.2). About 3.37 g l^{-1} of *S. platensis* biomass was measured at this phase (Fig. 3.6b). The increase in the production of *S. platensis* could have been due to the availability of more space, oxygen, and light to the culture flask.

Results described in Fig 3.6 (a, b) suggest that *S. platensis* was grown on different pH 8, 9, 10 and 11 but the maximum yield of *S. platensis* is obtain on 8< 9 >10>11. Thus 9 pH is optimum for the growth of *S. platensis*. Earlier results also demonstrated that optimum pH for maximal growth of *S. platensis* was 9 to 9.5 ranges (Belkin and Boussiba, 1971). *S. platensis* is considered to be an alkalophilic organism by nature (Grant *et al.,* 1990).



(a)



(b)

Fig. 3.6 Dry weight of *Spirulina platensis* on different pH in 500 ml flask (a) and in 1 litre flask (b)

Table 3.2: Physical and chemical composition of *S. platensis*

	Laboratory Culture	
Flask volume	500ml	1litre
Medium volume	250	500
Media used	Modified Zarrouk,s	Modified Zarrouk,s
Appearance	Fine powder	Fine powder
Colour	Green	Green
Odour and Taste	Mild and green vegetable	Mild and dark green vegetable
Bulk Density	1.25 g/l (on 10^{th} day)	3.7 g/l (on 12^{th} day)
Chemical composition		
Moisture	3-5%	3-5%
Ash	4%	5%
Proteins	60%	62%
Carotenoids	1.7 g/kg	1.7 g/kg
PC+APC+PF	2.06g/Kg	2.15g/Kg

It has been observed that the culture conditions viz medium (Ciferri 1983; Venkatraman and Mahadevaswamy, 1992), temperature (Torzillo and Vonshak, 1994; Deshnium., 2000), light intensity (Vonshak and Guy, 1992; Vonshak, 1997) and pH (Grant *et al.,* 1990; Belkin and Boussiba, 1971; Rafiqul *et al.,* 2003, 2005) etc. play vital role in biosynthesis of chemical constituents of the *S. platensis*. According to different efforts of optimization of optimal temperature for laboratory cultivation of *S. platensis* which ranges from 30-38^{0}C (Vonshak and Tomaselli, 2000) and the pH ranges between 9 - 9.5 (Rafiqul *et al.,* 2003, 2005) to obtain maximum yield of *S. platensis* in laboratory.

Changes in the pattern of gene expression have been described by characterizing the protein profile of *S. platensis* under various growth conditions. Three groups can be classified in response to various environmental stresses:

i) Protein whose expression remains relatively unaffected by variation in environmental factors

ii) Proteins whose synthesis is switched off or inhibited specifically when environmental conditions are changed

iii) Protein whose synthesis is increased or induced specifically under unfavorable environmental conditions

Proteins belonging to IIIrd category are called as stress proteins, which are of special interest because they perform important functions during adaptation to changed environment.

Chemical composition of cyanobacteria is known to be modified by composition of the culture medium (Mostert and Grobbelaar, 1981). Hence protein profile of *S. platensis* grown in Zarrouk's basal medium and Zarrouk's modified was compared using SDS-PAGE (Fig. 3.7). It has been found from results that *S. platensis* is capable to grow on ZM and ZB media and expressed and synthesized proteins. These proteins, however, differed in their molecular weights and sequence in which they are synthesized during change in the chemical composition of culture medium. There is only one protein that is expressed on Zarrouk's modified medium as compared to Zarrouk's basal medium. Protein, which was expressed only in Zarrouk's modified medium, is 21 KD, and the expressions of other proteins are similar on both

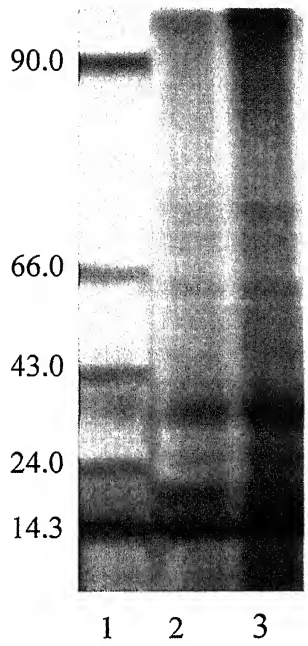


Fig. 3.7 Cellular protein profile of *Spirulina platensis*; protein markers (Lane 1); *Spirulina platensis* grown on ZB medium (Lane 2) and *Spirulina platensis* grown on ZM medium (Lane 3)

culture medium. The synthesis of the new proteins in response to culture medium plays an important role in the maintenance of vital cellular functions in cyanobacteria.

Zarrouk's modified medium having some trace element for the growth of cyanobacteria as compare to Zarrouk's basal medium is found to be better for the growth of *S. platensis*.

3.2 The effect of UV-B radiation on the morphology of *S. platensis*

S. platensis is a filamentous, photosynthetic, spiral shaped; multicellular cyanobacterium (Cifferi, 1983). The filament of *S. platensis* consists of cylindrical cells arranged in unbranched helicoidal trichomes. The helical structure of *S. platensis* varies with the species and even within the same species. *S. platensis* non-heterocystous filaments, composed of vegetative cells that undergo binary fission in a single plane, shows easily visible transverse cross-walls. The trichome, enveloped by a thin sheath and no akinetes formation is occurred in any phase of life cycle.

Structurally, the cyanobacteria have similarities with plastids (chlorophyll-containing bodies) of the algae and all higher plants. They also possess a mucilaginous sheath of cellulose fibrils varying in thickness from one genus to another. *S. maxima* and *S. platensis* are the most important species in this genus and among these exist taxonomic differences in filaments, vacuoles and external cover or capsule regularity of each filament (Tomaselli, 1997).

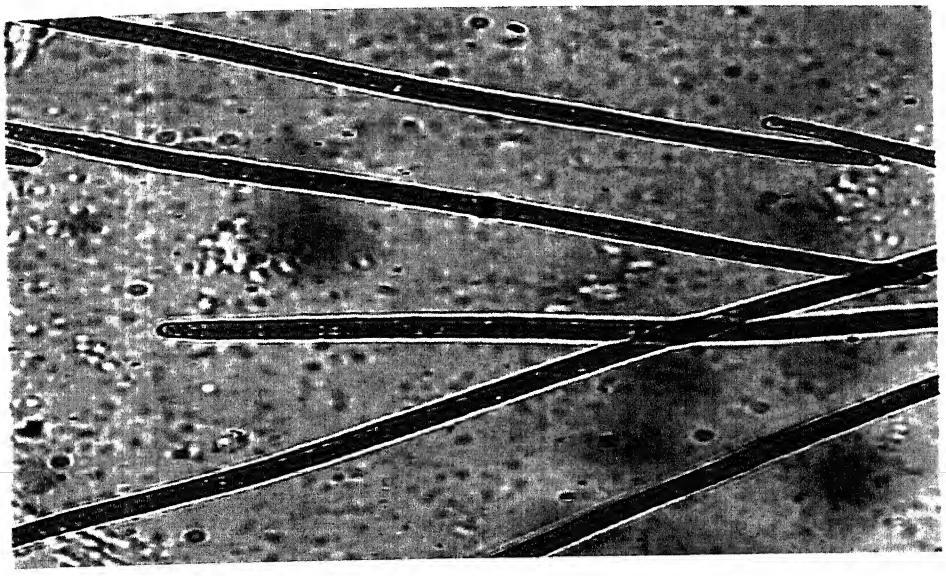
The pitch distances of trichome of *S. platensis* vary with the climatic condition like temperature and radiation (Jeeji Bai and Seshadri, 1980: Jeejibai, 1985; Lewin, 1980; Kebede, 1997). Under specific climatic condition the helical nature of *S. platensis* vary with availability at different profiles of the pond. The *S. platensis* available at the top are having fewer spirals as compared to intermediate and bottom layer of the pond. Therefore, the spiral nature of *S. platensis* is influenced by light.

Microscopical examinations of *S. platensis* were carried out by bright field, fluorescent and scanning electron microscopy

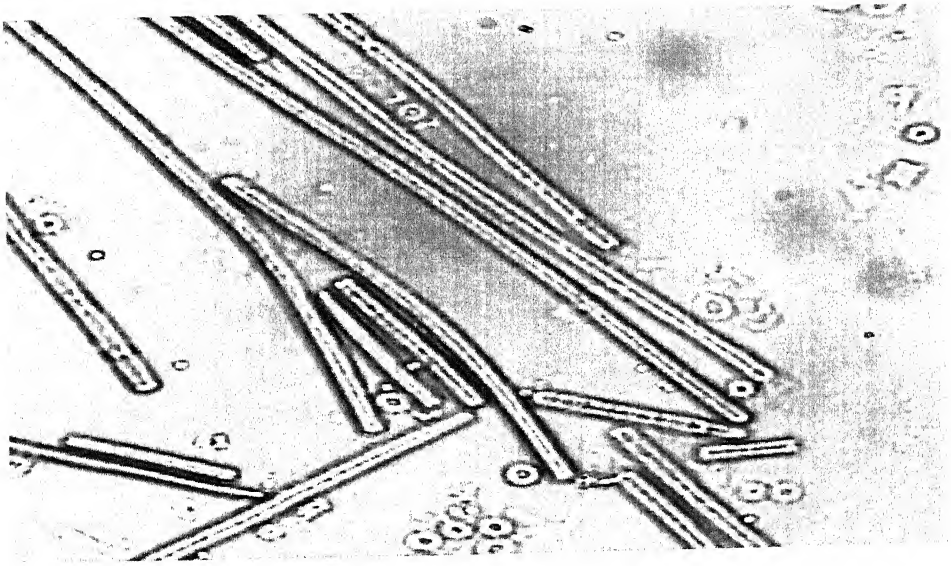
demonstrating significant change in the trichome of UV-B treated as compared to UV-B untreated counterpart.

A bright field microscopy shows significant change in the filament of *S. platensis* during UV stress. A morphological difference in the filaments was observed when *S. platensis* was irradiated with UV-B radiation (Fig. 3.8 a, b); showing changes in terms of granulation, pigmentation and both apical and terminal end of filaments. Fluorescent microscope results (Fig. 3.9 a, b) revealed that fluorescent emission of pigment in UV-B untreated *S. platensis* was higher as compared to UV-B treated cells due to presence of high level of fluorescent pigments. The surface scanning electron micrograph (Fig. 3.10a) of UV-B untreated *S. platensis* shows the smooth morphological structure with the appearance of ridges due to coverage of sheath. However, UV-B treated *S. platensis* shows distorted and straight morphological structure (Fig. 3.10b).

It is confirmed by various microscopical examinations (Fig. 3.8 a, b; 3.9a, b and 3.10a,b) that UV-B radiation affect the

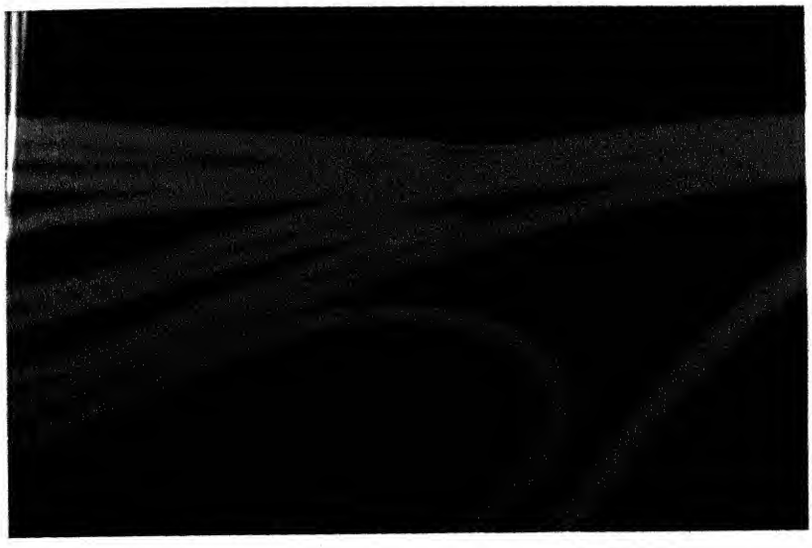


(a)



(b)

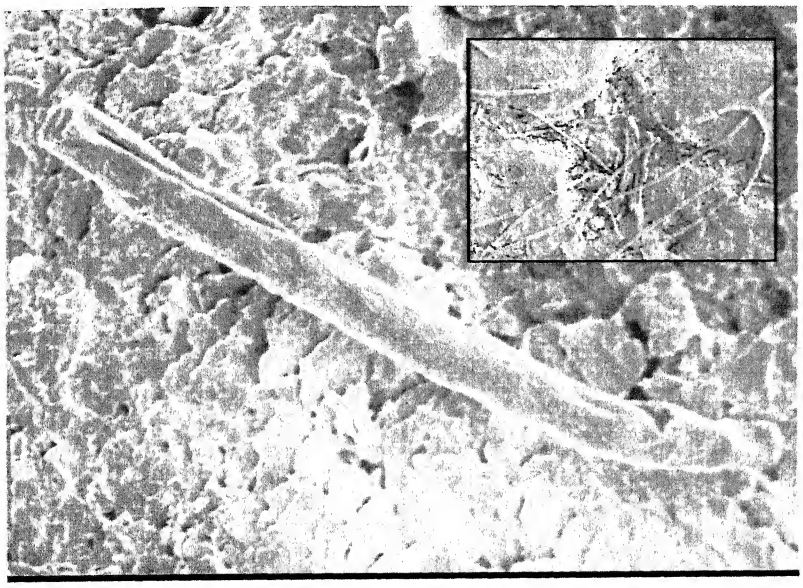
Fig 3.8 Bright field photomicrograph of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*



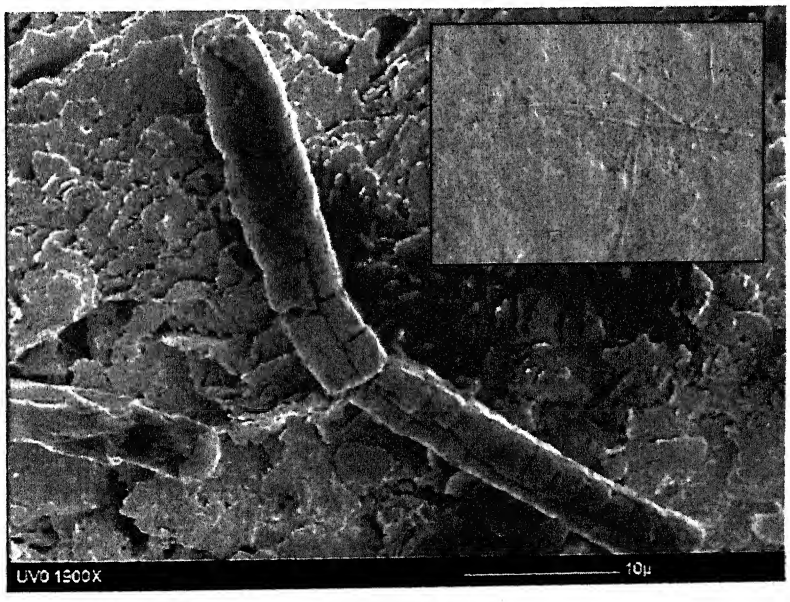
(a)

(b)

Fig. 3.9 Fluorescent photomicrograph of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*



(a)



(b)

Fig. 3.10 Scanning electron photomicrograph of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*

morphology of *S. platensis*. It is evident from previous studies that environmental factor (Van Eyelenburg, 1979) such as light, temperature, and salinity, can affect the helical structure (Jeejibai and Seshadri, 1980: Jeejibai, 1985; Lewin, 1980; Kebede, 1997); for example, the filaments change from straight to the typical helical shape when they are shifted to growth with a high intensity of visible light (Fox, 1996).

Recent studies suggested that UV-B treatment not only damaged photosynthetic light-harvesting complex of cyanobacteria and but also affect the ultrastructure (Hongyan *et al.,* 2005; Holzinger and Lutz, 2006). Stress induced morphological changes are reported in microorganism *Pseudomonas aeroginosa* (Cefali *et al.,* 2002), cyanobacteria (Rajgopal *et al.,* 2000) and algae (Holzinger and Lutz, 2006).

3.3 Effect of UV-B Radiation on the growth and protein profile of *S. platensis*

S. platensis is rich in ingredients that have nutrition and biomedical values (Mazo *et al.,* 2004; Khan *et al.,* 2005). *S. platensis* is known to have components having

immunomodulating (Baojiang *et al.,* 1994; Quershi *et al.*, 1995; Qureshi and Ali, 1996; Nemoto-Kawamura *et al.,* 2004), antiviral (Patterson, 1993; Hayashi *et al.*, 1994; Hernandez-corona *et al.,* 2002; Shih *et al.,*2003), anticancer (Lisheng *et al.,*1991; Mittal *et al.*, 1999; Liu *et al.,*2000; Dasgupta *et al.,*2001; Guan and Guo, 2002; Subhashini *et al.*, 2004), antioxidant (Jorjani and Amirani, 1978; Gorban *et al.*, 2000; Premkumar *et al.*, 2000; DasGupta *et al.,*2001; Wang *et al.,* 2001; Upasani and Balaraman, 2003; Chen and Zhou, 2003; Patel *et al.*, 2006), anti allergic (Qishen *et al.,* 1989), radioprotective (Moreno, 1997) and hypocholesterolemic (Nayaka *et al.,* 1988; Devi *et al.,* 1983) properties.

Cyanobacteria depend on solar radiation as the primary source of the energy in their natural environment. UV-B radiation has been a ubiquitous problem for life and particularly for the photosynthetic organisms including cyanobacteria (Capone *et al.,* 1997; Sinha *et al.*, 2001; Ferreira *et al.,* 2004). The potential threat to these cyanobacterial communities is the continuous solar ultraviolet-B (UV-B 280-315) radiation reaching the Earth's surface due to depletion of the stratospheric ozone layer (Blumthaler *et al.,*

1990; Crutzen, 1992; Kerr *et al.,* 1993; Arrigo, 1994; Neale *et al.*, 1998; Lubin *et al.,* 1995; Sinha *et al.,* 2003).

Ultraviolet radiation is injurious to a wide variety of biological systems. Biological effect of UV-B radiation includes DNA damage in most organism (Harm, 1980; Karentz *et al.,* 1991, 1991b; Gour *et al.,* 1997), killing of bacteria (Kumar *et al.,* 2004), pigment bleaching and photoinhibition of photosynthesis in cyanobacteria (Cullen *et al.*, 1992; Bhandari and Sharma, 2006), inhibition of motility (Donkor and Hader, 1995), inhibition of nitrogenase activity (Sinha *et al.,* 1996; Tyagi *et al*, 2003), inhibition of heterocyst formation in some cyanobacteria (Sinha *et al.,* 1996) and morphological changes (Wu *et al.,* 2005; Hongyan *et al.,* 2005; Holzinger and Lutz, 2006). Cyanobacteria show wide variation in tolerance to UV-B and posses a variety of defense strategies, such as avoidance of brightly lit habitats (Xiong *et al.,* 1997; Rajgopal *et al.*, 2005), production of UV-absorbing compounds, such as micosporine-like amino acid and scytonemin (Garcia-Pichel *et al.,* 1993; Sinha *et al.,* 2002; Sinha *et al.,* 2003; Rezanka *et al.*, 2004), and active repair or de novo synthesis of DNA (Sass *et al.,* 1997; Sinha and Hader, 2002). Photodynamic reactions are

potential mechanisms by which ultraviolet radiation induces damage to living cells (Ito, 1983). The high energy of short wavelength photons absorbed by chromophore molecules can lead to the formation of singlet oxygen or free radicals that are known to destroy membranes and other cellular components (Benson *et al.,* 1992; Alscher *et al.,* 1997; Mackerness *et al.,* 1999; Vega and Pizarro, 2000).

In the present study we have investigated the impact of UV-B radiation on growth (in term of specific growth rate) and changes in biological compounds like chlorophyll, protein, carbohydrate content, and physiological parameters like nitrate uptake, nitrite uptake, nitrate and nitrite reductases in *S. platensis*.

Specific growth rate (μh^{-1}) for UV-B untreated *S. platensis* was 0.065, and for UV-B treated *S. platensis* was 0.049 (Table 3.3). The maximum bulk densities were 1.25 gl^{-l} for UV-B untreated *S. platensis* and 1.10g l^{-l} for UV-B treated *S. platensis* (Fig. 3.11). The specific growth rate and biomass were found to be lower for the UV-B treated cells of *S. platensis* as compared to UV-B untreated counterpart.

Table 3.3: Effect of UV-B stress on growth: in terms of specific growth rate (μh^{-1}), dry weight (g l^{-1}), chlorophyll content (in %), protein content (in %), carbohydrate content (in %), NR activity (μmol NO_2^- produced mg^{-1}protein min^{-1} and NiR activity (μmol nitrite reduced mg^{-1}protein min^{-1}) of UV-B treated and UV-B untreated *S. platensis*.

S. No.	Growth Parameters	UV-B UV-B untreated *S. platensis*	UV-B treated *S. platensis*
1	Specific growth rate	0.065	0.049
2	Dry weight	1.25	1.10
3	Chlorophyll content	1.82%	1.41%
4	Protein content	61.0%	51.5%
5	Carbohydrate content	21.97%	34%
6	NR activity	8	3.8
7	NiR activity	5	2.25

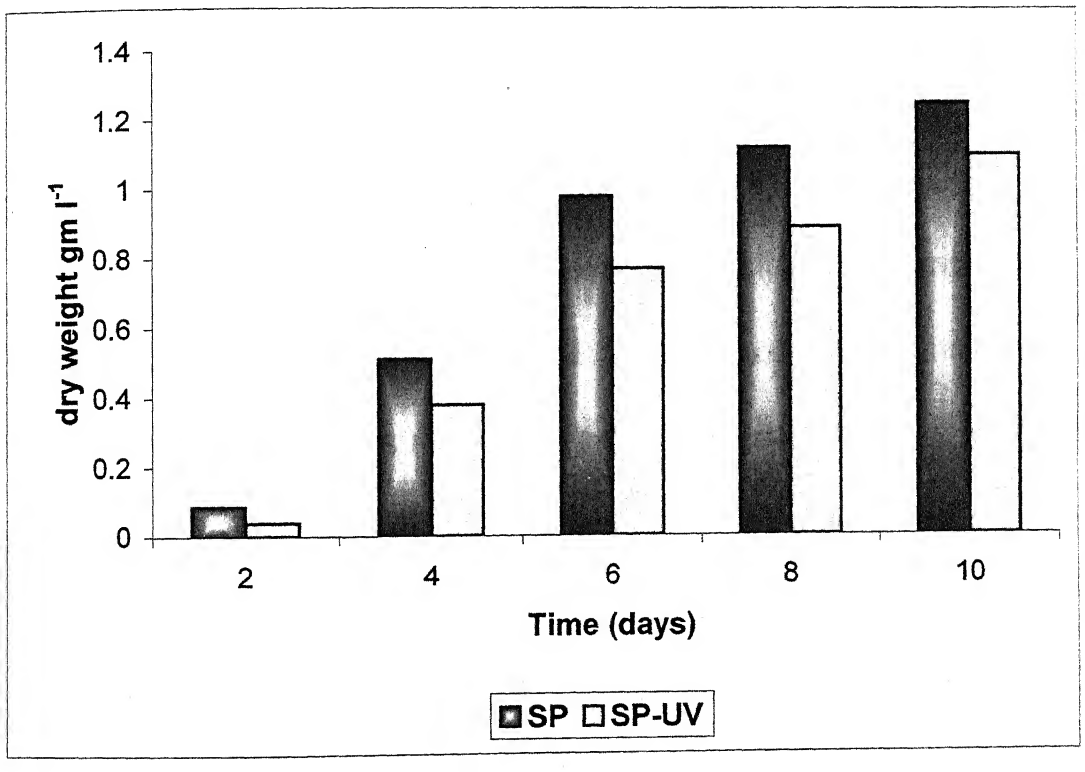


Fig. 3.11 Dry weight of *Spirulina platensis* in response to UV-B stress

The chlorophyll *a* content was achieved 1.82% for UV-B untreated cells of *S. platensis* and 1.41% for UV-B treated cells of *S. platensis* (Fig. 3.12). Result shows that chlorophyll *a* content is reduced significantly for UV-B treated *S. platensis* as compared to UV-B untreated counterpart.

Protein content achieved for UV-B untreated cells of *S. platensis* was 61.0% and for UV treated cells was 51.5% (Fig. 3.13). Carbohydrate content achieved for UV-B untreated *S. platensis* was 21.97%, and for UV-B treated cells of *S. platensis* was 34% (Fig. 3.14). Result shows that protein content was higher (9.5%) in UV-B untreated *S. platensis* as compared to UV-B treated counterpart. It is evident that the carbohydrate content increased 12% in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. Thus UV-B radiations stimulate the synthesis of carbohydrate for the protection of cell from stress.

Protein content and chlorophyll content is reduced under stresses (Sinha *et al.,* 1995; Vonshak, 1997, 1996). Carbohydrate synthesis is stimulated under stress (Tomaselli *et*

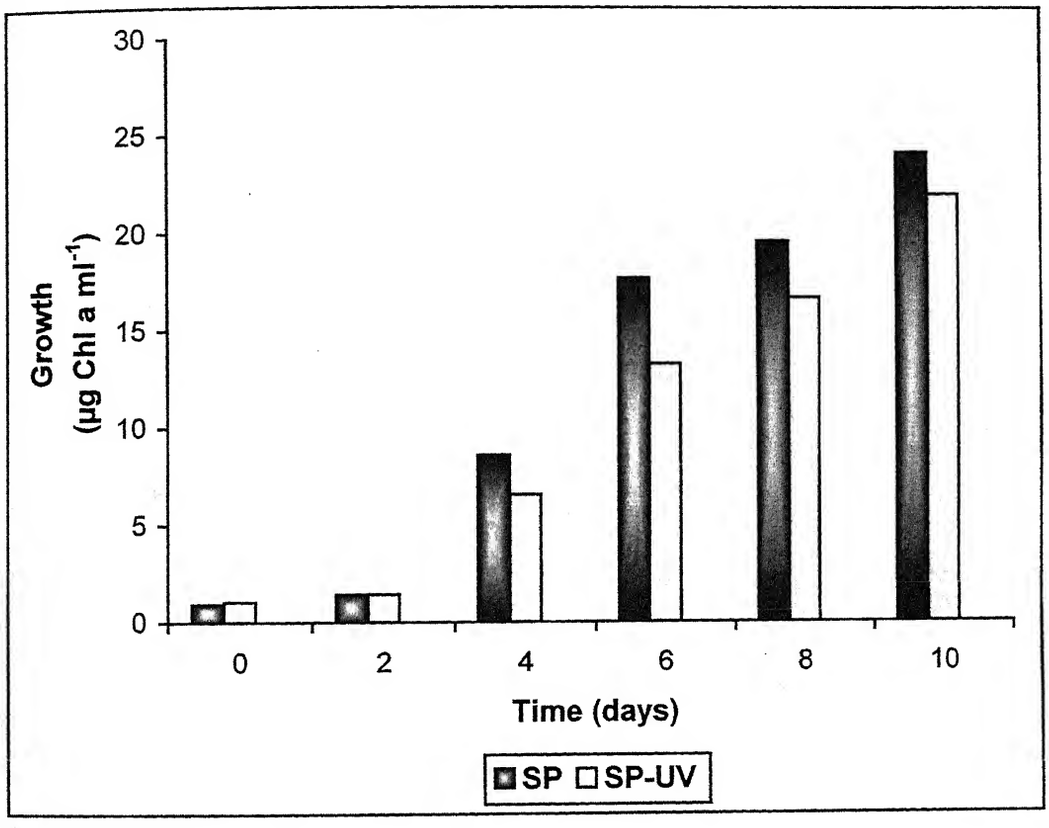


Fig. 3.12 Growth (in terms of chl *a*) of *Spirulina platensis* in response to UV-B stress

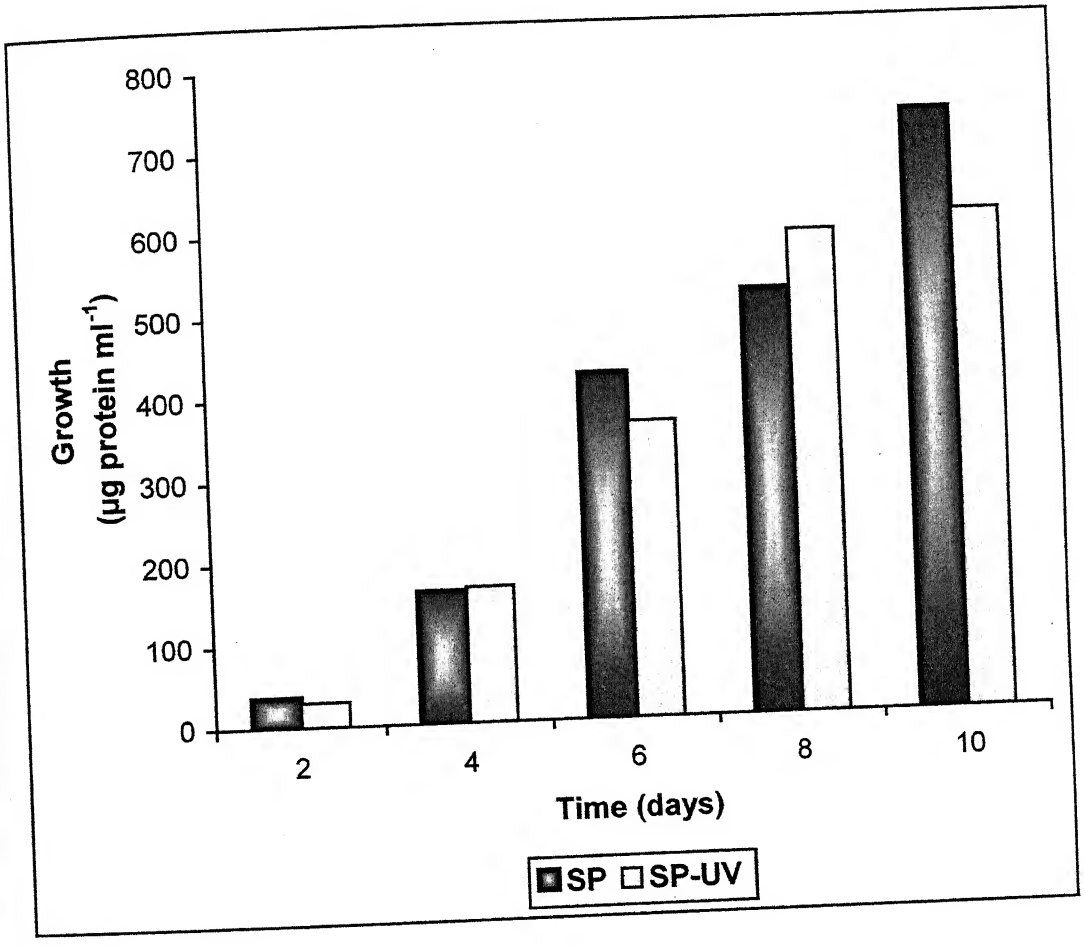


Fig. 3.13 Growth (in terms of protein) of *Spirulina platensis* in response to UV-B stress

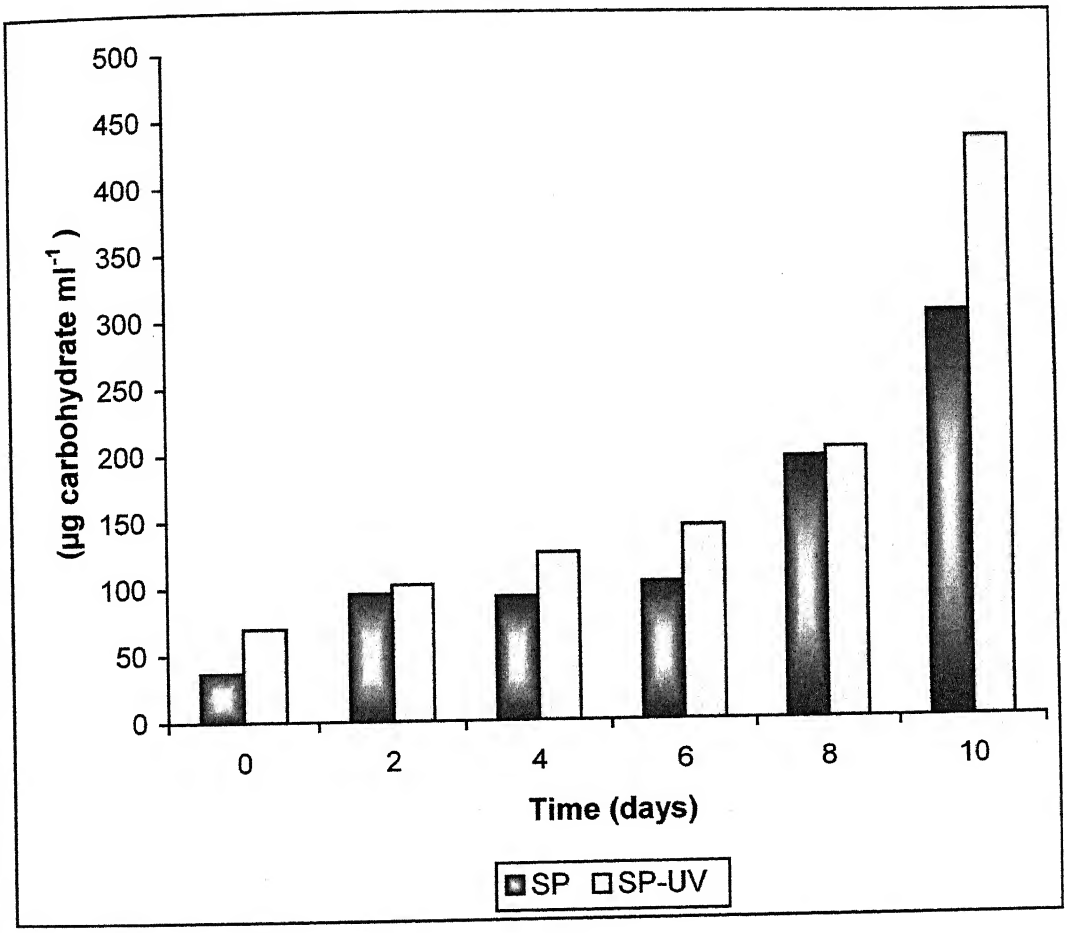


Fig. 3.14 Effect of UV-B stress on carbohydrate content of *Spirulina platensis*

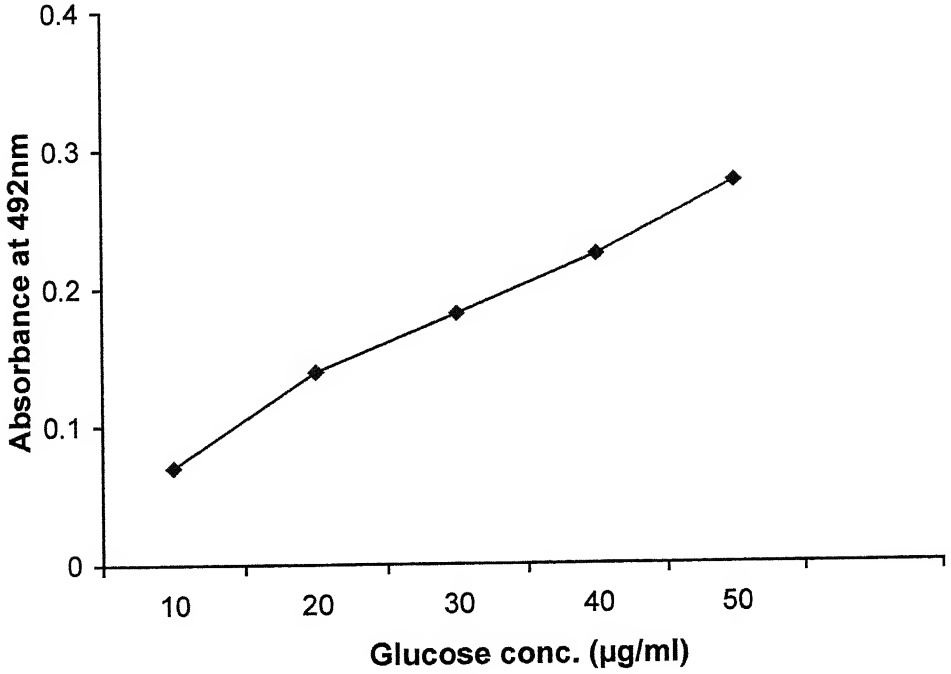


Fig.3.15 Carbohydrate standard curve (glucose)

al., 1987). Thus stress cells have a lower biosynthesis capacity for protein but higher biosynthesis capacity for carbohydrate.

Cifferi (1983) and Rafiqul *et al.,* (2005) suggested that the culture conditions, temperature, light intensity, irradiation and pH etc. are known to change the biochemical composition of the cyanobacterium *S. platensis*. Protein content of *S. platensis* is grown commercially for health food, which may range from 55 to 70% dry weight (Belay and Ota, 1993). UV-B radiation affects cyanobacterial photosynthesis and nitrogen metabolism is also studied in cyanobacteria (Newton *et al.,* 1979; Gour *et al.,* 1997).

NO_3^- and NO_2^- is probably the most abundant source of nitrogen for cyanobacterial nutrition. The first step in the metabolism of any nutrient by cyanobacteria is the entrance of that nutrient into the cell with the help of specialized uptake systems. The assimilation of NO_3^- by cyanobacteria involves NO_3^- uptake and reduction of intracellular NO_3^- (via NO_2^-) to NH_4^+, which is incorporated into organic compound (Manzano *et al.*, 1976). Nitrate metabolism in cyanobacteria suggested that the uptake of NO_3^- and NO_2^- is an ATP-dependent process, which takes place

through a membrane, bound permease system (Singh *et al.*, 1996a). The NO_3^- uptake system is mainly dependent on light as the dark incubated cyanobacterial cells showed drastic decline in its NO_3^- accumulating ability as compared to its light grown counterpart (Singh *et al.*, 1996b).

Fig. 3.16 showed nitrate uptake pattern in *S. platensis*. It is evident from results that the rate of nitrate uptake for UV-B treated cells of *S. platensis* was lower as compared to UV-B untreated *S. platensis*. Nitrate uptake pattern was characterized by a faster uptake rate for first 120 min. followed by a slower uptake at least up to 3 hrs. Fig. 3.17 showed nitrite uptake pattern in *S. platensis*. It is evident from results that the rate of nitrite uptake for UV-B treated cells of *S. platensis* was lower as compared to UV-B untreated *S. platensis* (Fig. 3.17). The rate of uptake of nitrate was found to be more as compared to nitrite under similar conditions. It is evident from the results (Fig. 3.16, 3.17) that UV-B untreated cells of *S. platensis* had high affinity for NO_3^- and NO_2^- as compared to the UV-B treated cells of *S. platensis*. An UV-B radiation is affected nitrate, nitrite uptake and NR activity of *Spirulina platensis*.

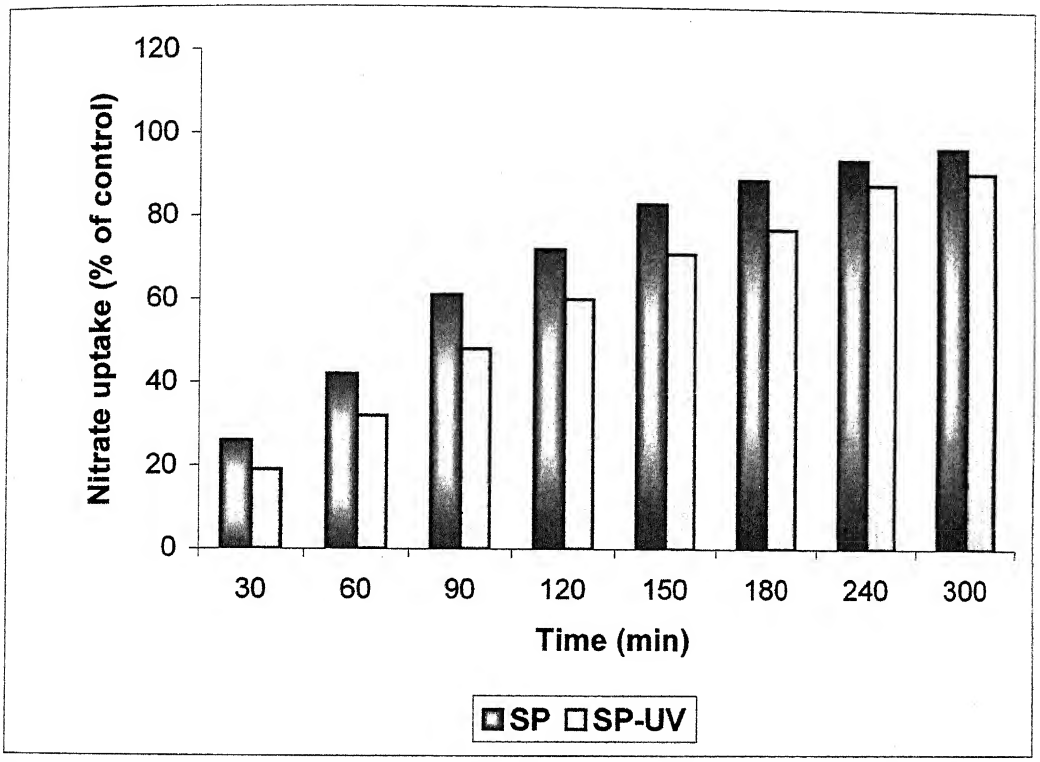


Fig. 3.16 Effect of UV-B stress on nitrate uptake of *Spirulina platensis*

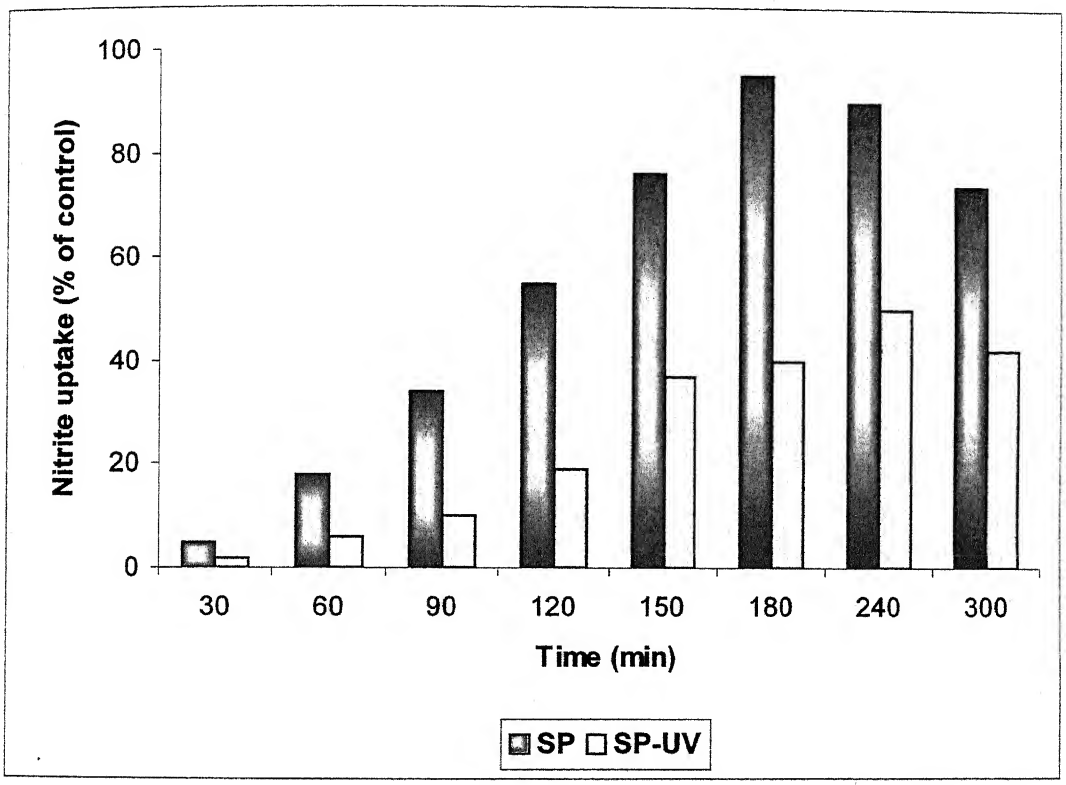


Fig. 3.17 Effect of UV-B stress on nitrite uptake of *Spirulina platensis*

Further to determine the correlation between nitrogen-metabolizing enzymes, nitrate reductase (NR), nitrite reductase (NiR) and NO_3^- and NO_2^- uptake, were also studied in both UV-B treated and UV-B untreated *S. platensis*. It is evident from the data that the UV-B treatment inhibited both NR and NiR activity by more than 50% in UV-B treated cells of *S. platensis* as compared to UV-B untreated *S. platensis* (Table 3.3).

It has been reported that UV-B exposure has a deleterious effect on the photosynthetic apparatus leading to the reduction in the supply of ATP and $NADPH_2$ (Kulandaivelu and Noorudeen, 1983). As such, disruption of cell membrane and /or alteration in thylakoid integrity as a result of UV-B radiation may partly damage the photosynthetic apparatus (Vu *et al.,* 1981). Thus, there is a possibility that the inhibition in NR activity might be due to the reduced supply of reductants and energy following the UV-B treatment.

UV-B radiation induced changes in nitrate metabolism are reported in *Anacystis nidulans* (Sinha *et al.,* 1995; Gour *et al.,* 1997) and green algae *Chlorella vulgaris* (Rai and Rai, 1997). Singh and Singh (2000) also suggested that high light stress

induced alterations in the nitrogen assimilatory enzymes in *S. platensis*.

Thus, UV-B radiation not only affects the biomass and biological compounds like chl a content, protein content, carbohydrate content, but also affects physiological parameters like nitrate uptake, nitrite uptake, nitrate reductase and nitrite reductase activity in *S. platensis.* This study is useful to study the impact of UV-B radiation on biomass and biological compounds of *S. platensis.* It will also be useful for enhancing potential of cyanobacteria in biotechnology.

3.4 Isolation, purification and Characterization of thylakoid membrane from *S. platensis* under UV-B stress

Cyanobacteria are unique prokaryotes due to the presence of distinct intracellular membrane system (Stanier, and Cohen, 1977; Gantt, 1994). The fundamental membrane structure of the cyanobacterial cell is the thylakoid membrane (Nomura *et al.,* 1995; Omata and Murata, 1983; Norling *et al.*, 1997; Norling *et al.*, 1998), a peptidoglycan layer between them (Jost, 1965; Murata *et al.,* 1981; Omata and Murata, 1983; Fujita *et al.,* 1994: Allnutt,

1996: Meijer *et al.,* 1999) and lipopolysaccharide is a major constituent of the outer membrane (Weise *et al.,* 1970; Omata and Murata, 1983; Neisser *et al.*, 1994; Fujita, 1996; Meijer *et al.,* 1999; Zak *et al.*, 2001). Cyanobacteria have the thylakoids, which is the site for both photosynthesis and respiration (Peschek *et al.*, 1988; Gantt, 1994; Cooley and Vermaas, 2001).

Thylakoid membrane is photosynthetically active membrane found in the cyanobacteria. Rapid adaptations to number of environmental factors are accompanied by changes in the lipid and protein content of thylakoids. Thus regulation of synthesis and assembly of all these elements is required to ensure the optimal function of this membrane (Vothknecht, 2001; Vothknecht and Soll, 2005).

The envelope and thylakoid membrane of cyanobacterial cells have been separated by using lysozyme treatment followed by French Pressure and separation using sucrose density gradient with slight modification of the method as described by Omata and Murata (1983) and Fluda *et al.,* (1999a). Membrane plays an important role during Na^+ transport across the membrane (Apte

and Thomas, 1986; Apte and Haselkorn, 1990; Ramani and Apte, 1997; Fluda *et al.,* 1999a).

LC-MS has revolutionized the biological science, and now biological macromolecules are mass measured with great accuracy and highly resolved spectra reveal subtle molecular heterogeneity (Whitelegge *et al.,* 1998).

In present study, we investigated comparative analysis of the thylakoid membrane in UV-B untreated and UV-B treated *S. platensis*. The separation and characterization of the thylakoid membrane in *S. platensis* under UV-B stress has undergone tremendous changes in its thylakoid membrane morphology, pigment; chl a, absorption spectrum of chl a, fluorescent spectrum and protein profile.

The cells of *S. platensis* were found to be very resistant to mechanical treatment and were efficiently disintegrated only after freezing and thawing followed by lysozyme treatment at 37^0C overnight. This was followed by French pressure. Unbroken cells were removed by centrifugation at 18,000 rpm for 30 min. Thylakoid membrane were harvested at 40,000xg for 90 min. on

the sucrose gradient from 30 to 90% (w/v) and were collected at interface between 39% and 50% with a light green band. Fig. 3.18 shows the light green band of thylakoid membrane of *S. platensis*.

A bright field microscopy result shows significant changes in the thylakoid membrane of UV-B treated *S. platensis* as compared to UV-B untreated counterpart (Fig. 3.19 a, b). It is evident that thylakoid membranes of *S. platensis* showed high chl *a* content and thickening on the outer side of the membrane as compared to UV-B treated counterpart. It is evident from result (Fig. 3.19 b) that under UV-B stress the membrane of *S. platensis* become partially distorted.

It is evident from the result of the absorption spectrum of thylakoid membrane of UV-B treated *S. platensis* that chl *a* content of thylakoid membrane was decreased as compared to UV-B untreated counterpart (Fig. 3.20 a, b). A dominating peak of thylakoid membrane was recorded at 660 nm that confirms the purity of membrane. Occurrence of this peak also suggests the presence of chl *a* in the thylakoid.

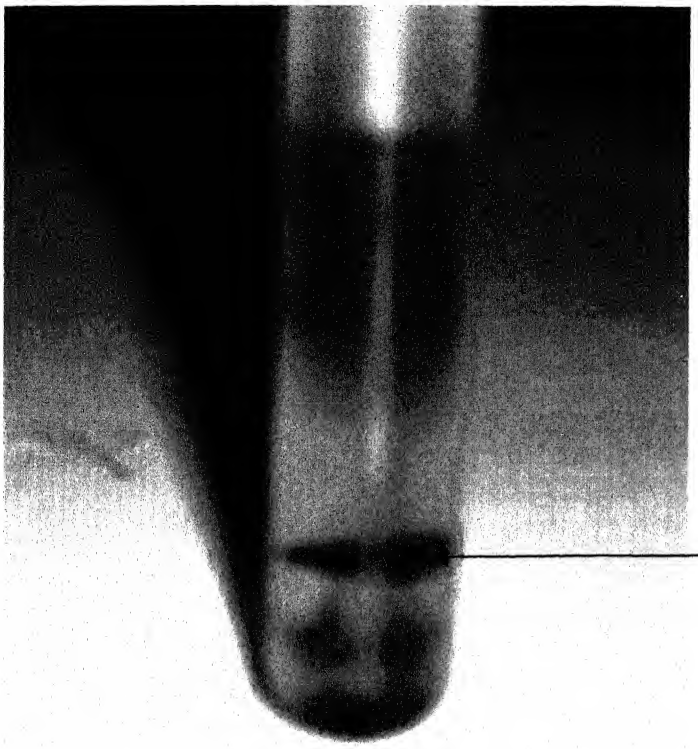
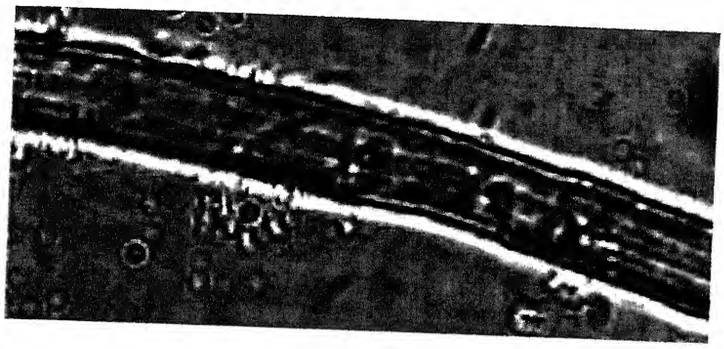
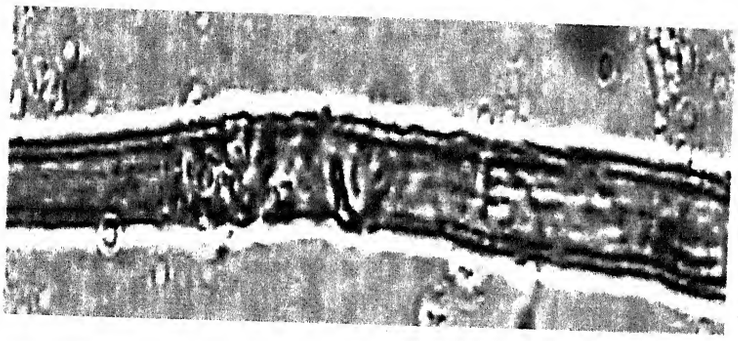


Fig. 3.18 Thylakoid membrane of *Spirulina platensis* showing dark green band

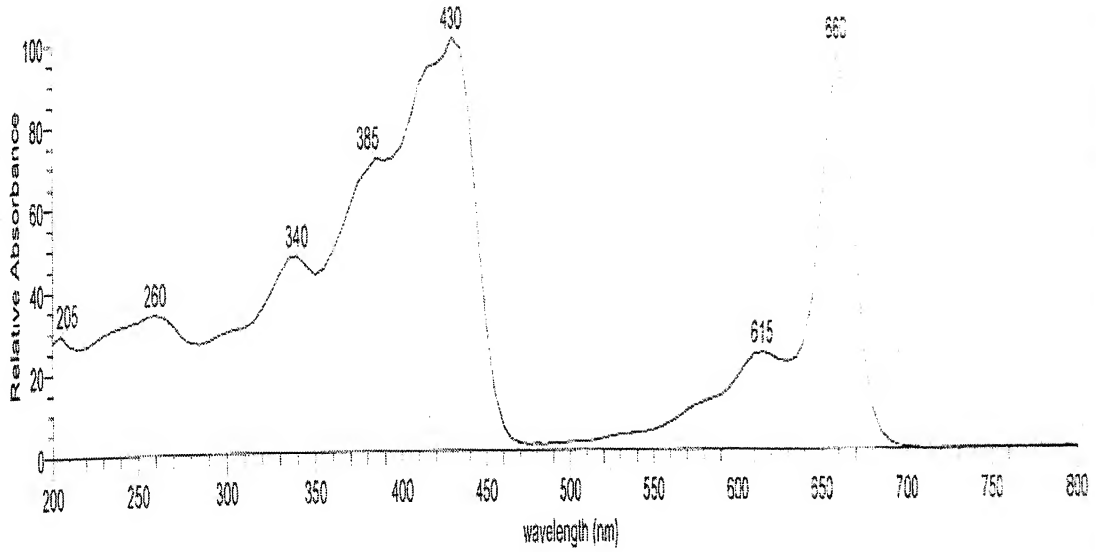


(a)



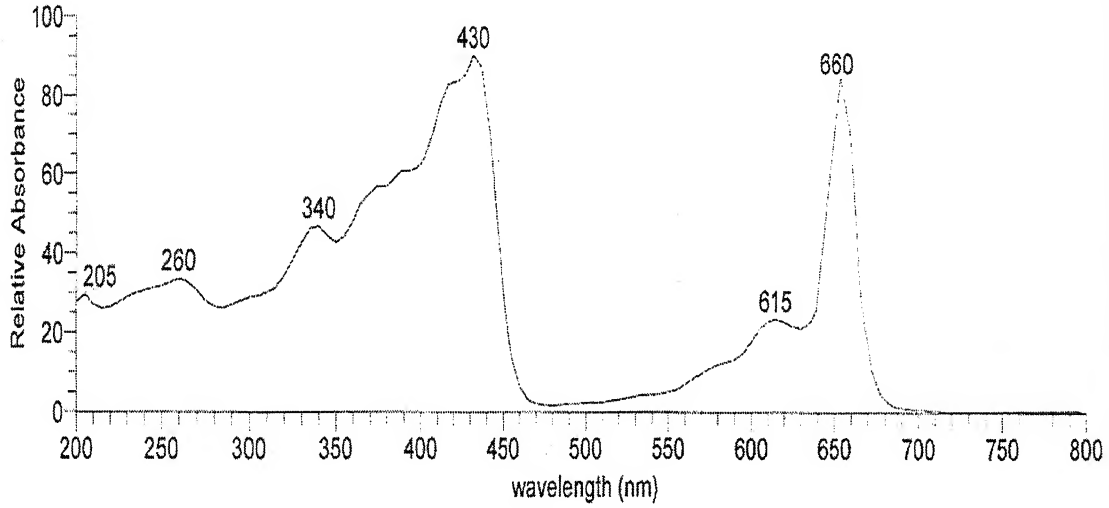
(b)

Fig. 3.19 Bright field photomicrograph of thylakoid membrane of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*



(a)

wavelength (nm)

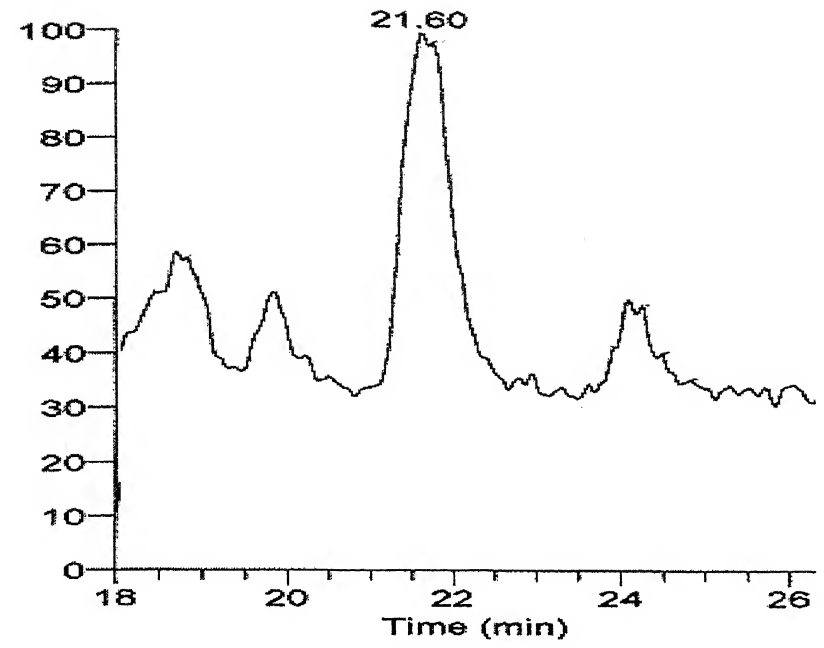


wavelength (nm)

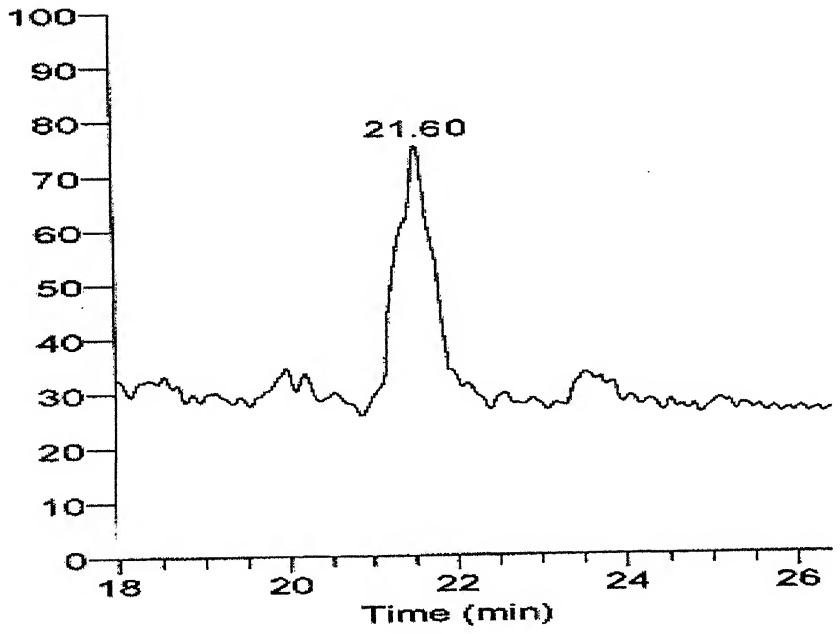
(b)

Fig 3.20 Absorption spectra of chl *a* at 660nm of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*

This is the first report, which deals with the separation, and characterization of pigment of thylakoid membrane of UV-B treated *S. platensis* through LC-MS. We used ODS column, utilizing methanol and 2 propanol as mobile phase by using APCI method for the analysis of pigment chl *a* in *S. platensis*. LCMS analysis of pigment chl *a* in UV-B treated *S. platensis* showed alter chl *a* level as compared to UV-B untreated counterpart (Fig. 3.21a, b). The level of chl *a* in UV-B untreated *S. platensis* was higher than that of UV-B treated counterpart (Fig. 3.21a, b). Chl *a* was separated and purified from other pigments through an ODS coloumn and then subjected to PDA (photodiode array) detector. Chlorophyll is a light harvesting protein, which serve as photosynthetic antenna through absorption and funneling of excitation energy to photosystem II and I. Chl *a* is a porphyrin derivative having molecular weight 893.49 (Fig 3.22). Due to Na adduct of molecular ion ($M+Na^+$), m/z ratio of chlorophyll altered to 915 (Fig 3.23 a, b). Chlorophyll had a specific absorbance peak at 660 nm. Our LC-MS coupled with PDA detector result demonstrated that Chl *a* level is decreased in UV-B treated *S. platensis* as compared to UV-B untreated counterpart.



(a)



(b)

Fig. 3.21 Part of total ion chromatogram of LC-MS analysis of pigment chl *a* of UV-B untreated (a) UV-B treated (b) *Spirulina platensis*

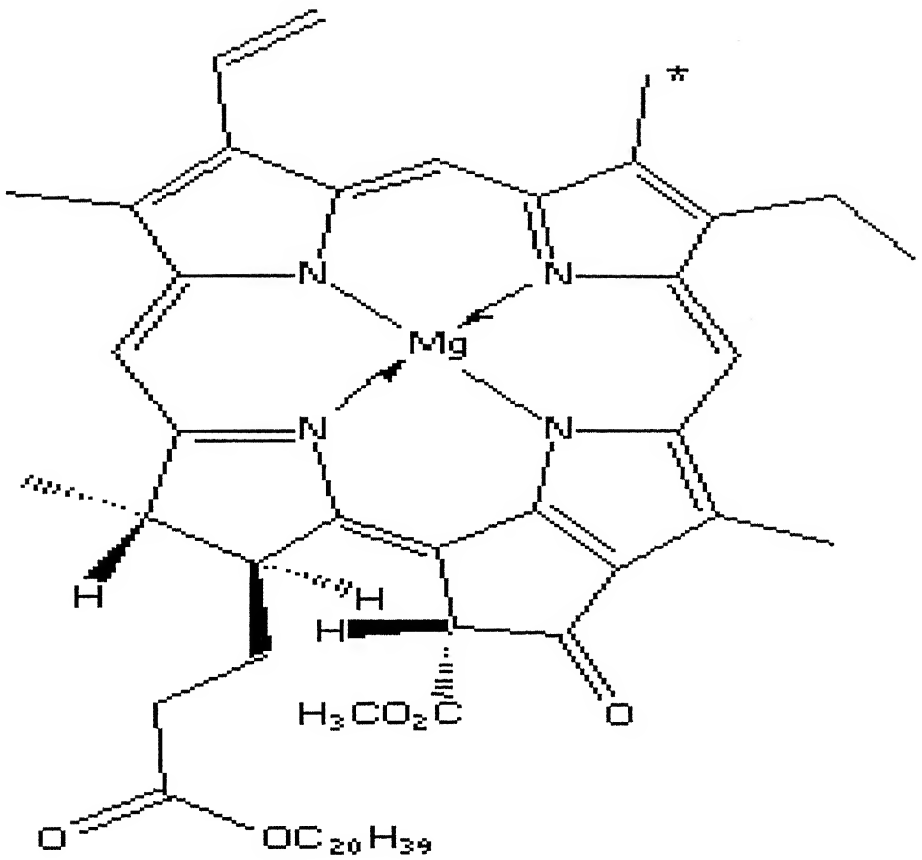
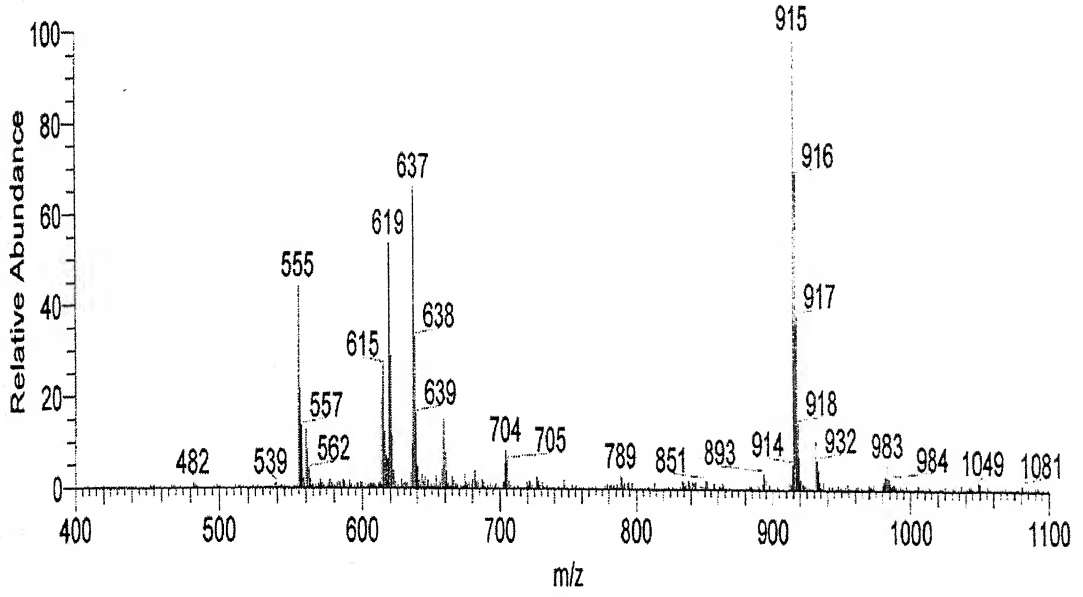
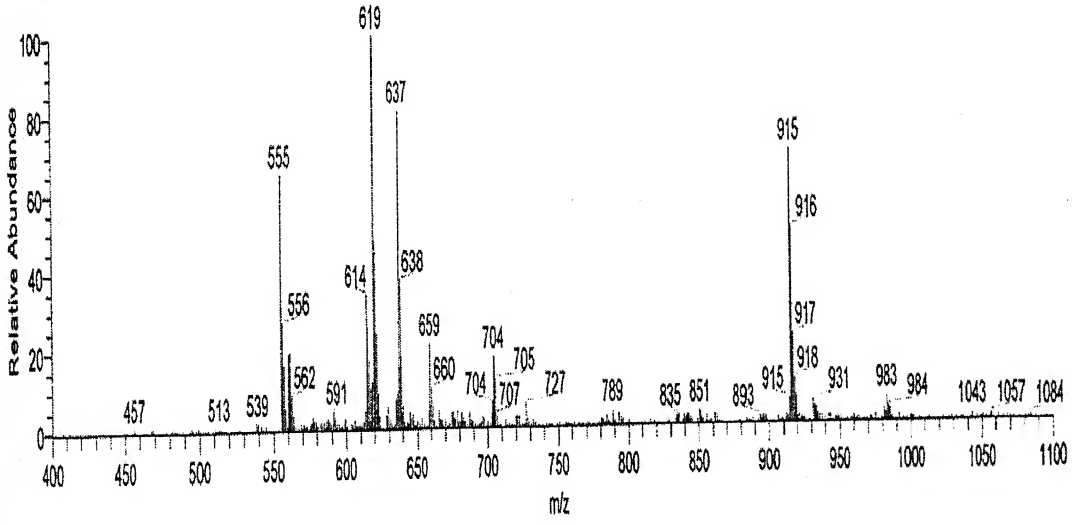


Fig. 3.22 Chemical structure of chlrophyll *a* having a molecular weight 893.49



(a)



(b)

Fig. 3.23 Mass spectra of chl *a* in UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*

Environmental factors such as UV radiation are known to affect photosynthesis in both cyanobacteria (Vass *et al.*, 2000). Changes in the pattern of gene expression have been described by characterizing the protein profile of *S. platensis* under various growth conditions. Protein whose synthesis is induced under stress is called stress proteins, which are of special interest because they perform important functions during adaptation to changed environment.

Cyanobacterial sp. is capable to grow under UV-B stress but some stressed proteins are over expressed and some disappeared. It is evident from the SDS-PAGE electrogram of *S. platensis* (Fig. 3.24) that intact trichomes of *S. platensis* are exposed to UV-B radiations, which affect the protein profile of *S. platensis*. This UV-B exposure results in alterations in the pigment-protein complexes CP47 and CP43 are affected by UV-B irradiation. Furthermore, 94kD and 20KD protein band is only appeared in the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of UV-B-exposed thylakoids membrane of *S. platensis* as compared to UV-B untreated

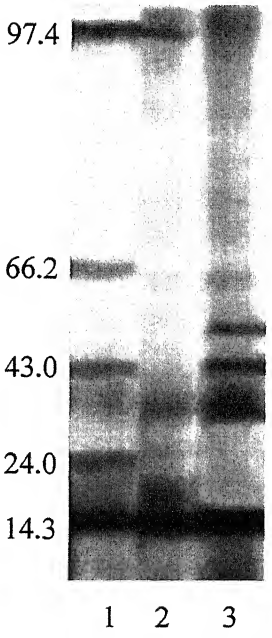
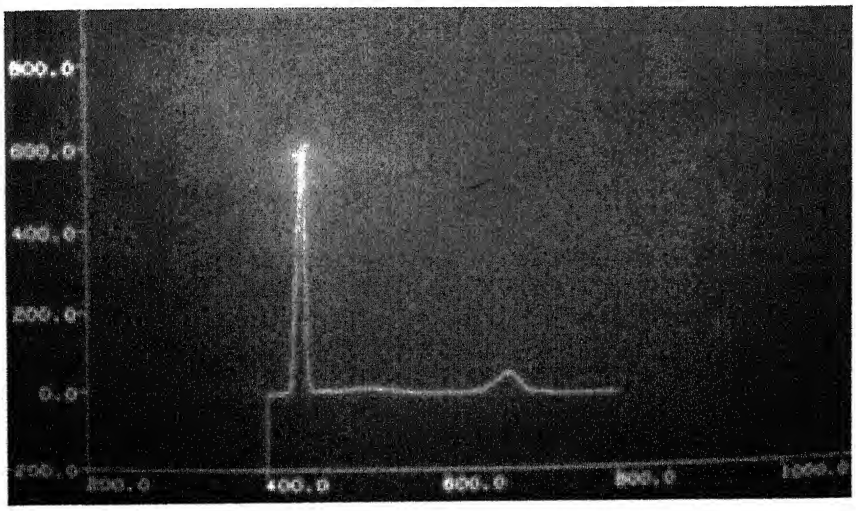


Fig. 3.24 UV-B induced modification of thylakoid membrane protein synthesis in *Spirulina platensis*; protien markers (Lane 1); UV-B treated (Lane 2) and UV-B untreated (Lane 3)

counterpart. This 94 kDa and 20KD protein appears not to be newly induced by UV-B exposure, but could possibly have originated from the UV-B-induced cross-linking of the thylakoid proteins.

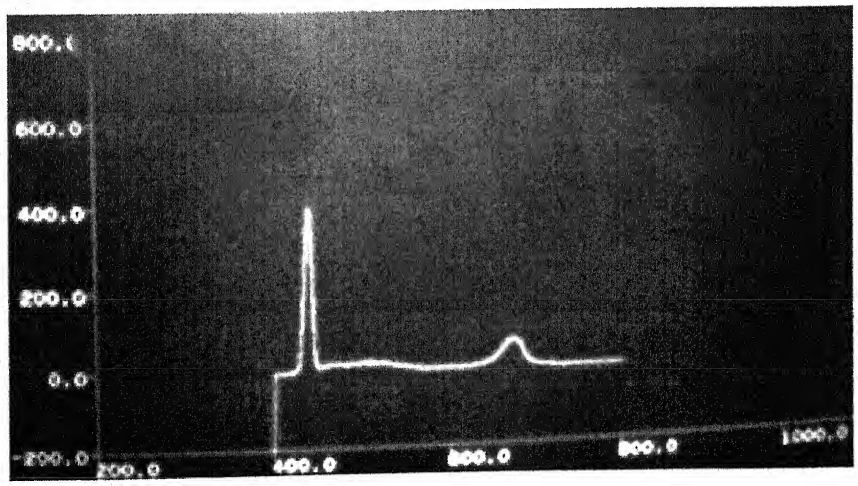
Several studies demonstrated that thylakoid membrane proteins were affected by UV irradiation (Rajgopal *et al.,* 1998, 2000). D1 protein of thylakoid membrane was showed as a sensitive protein to environmental stress condition: under various unfavorable conditions like drought, nutrition deficiency, heat, chemical stress, ozone fumigation as well as UV-B and visible light stresses (Giardi *et al.,* 1997; Campbell *et al.,* 1998; Vass *et al.,* 2000). Similar conclusions have been reached in higher and lower plants during photoinhibitory stress (Rintamaki *et al.*, 1994) Proteins from thylakoid membrane have studied in cyanobacterium *Synechocystis* sp. PCC 6803. (Wang and Chitnis, 2000; Vass *et al.*, 2000).

The fluorescence emission spectrum of the chlorophyll of thylakoids membrane was monitored in UV-B treated and UV-B untreated *S. platensis* (Fig. 3.25 a, b). Fluorescence emission



Wavelength (nm)

(a)



Wavelength (nm)

(b)

Fig. 3.25 Fluorescence emission spectra of the chl *a* of thylakoids membrane of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*

spectrum of the chlorophyll was taken at room temperature. Present results clearly demonstrate that prolonged exposure of UV-B radiation alters the fluorescence emission spectral profile of the thylakoid membranes of *S. platensis* as compared to UV-B untreated counterpart (Fig. 3.25 a, b). Thus, it is evident that UV-B radiations alter the fluorescence emission spectral profile of thylakoid membrane of *S. platensis*.

Rajgopal *et al.,* (1998) suggested that prolonged exposure of UV-B irradiation affects the chl a-protein complexes of the thylakoid membranes of cyanobacteria. Thus, it is clear that chlorophyll *a* antennae of *S. platensis* are significantly altered by UV-B radiation. Similar conclusions have been reached in higher and lower plants during photo-inhibitory stress (Ohad *et al.,* 1984; Rontamaki *et al.,* 1994; Garnier *et al.,* 1994) and in cyanobacteria (Krause and Weis, 1991; Rajgopal *et al.,* 2000).

3.5 Effect of UV-B radiation on fatty acids profile of *S. platensis*

S. platensis possesses diverse biological activities and having a long history of use as food supplement (Belay, 1997;

Cifferi, 1983). It is the most commonly used species of cyanobacteria as concentrated natural source of nutrition and biomedical values, e.g. essential fatty acids (Hwang, 1989; Pascaud, 1993; Huang and Mills, 1996; Cohen *et al.*, 1987; Mahajan and Kamat, 1995), and so on.

Fatty acids are composed of a long hydrocarbon chain and a terminal carboxylate group and a great variety of fatty acids exit in nature. In biological systems, fatty acids are mostly encountered as components of lipids. The lipids that contribute to the structure and function of biological membrane are called structural lipids. Cyanobacteria and some bacilli can introduce double bonds into fatty acids by using oxygen-dependent desaturases enzyme (Bloomfield and Bloch 1960; Shanklin and Cahoon, 1998).

Fatty acid composition is known to be affected by changes in growth rate (Liang *et al.*, 2006) and by the concentration of substrates in the medium. The effects of changes in growth conditions on lipid accumulation and its composition have been reviewed by Rattrary *et al.*, (1975).

Fatty acid has got a property to become less saturated at lower temperature and become more unsaturated at higher growth temperature. This is apparently due to the lower solubility of oxygen at higher temperature resulting in low concentration of unsaturated fatty acid, since low oxygen concentration causes a decrease in the desaturation of the saturated fatty acids. Oxygen is required for the conversion of stearic acid to oleic acid and to linolenic acid etc. Changes in pH, have little effect on growth and consequently on the fatty acids. Organisms accumulate more lipids in the medium containing organic source rather than inorganic source of nitrogen (Blinc and Hocevar, 1953; Witter *et al.,* 1974). Temperature is a crucial parameter, since it may have a substantial impact on fatty acid composition itself (Wada *et al.*, 2000; Deshnium *et al.*, 2000) as well as on the dynamics of repair mechanisms (Roos and Vincent, 1998).

Polyunsaturated fatty acids (PUFAs) play important roles as structural components of membrane phospholipids. Polyunsaturated fatty acids have made up an essential part of the human diet (Pascaud and Brouard, 1991; Pascaud, 1993). They are nutritionally important for various reasons. PUFA such as GLA

is found in higher plants like evening primrose, black currant and borage as well as in cyanobacteria (Huang *et al.*, 1982) and fungi. In plant oils GLA is present either in low conc. or is associated with other undesirable fatty acids, from which large scale purification could be very expensive. *S. platensis* is unique that contains substantial quantities of GLA (Huang *et al.*, 1982; Huang and Mills, 1996; Cohen *et al.,* 1987; Mahajan and Kamat, 1995).

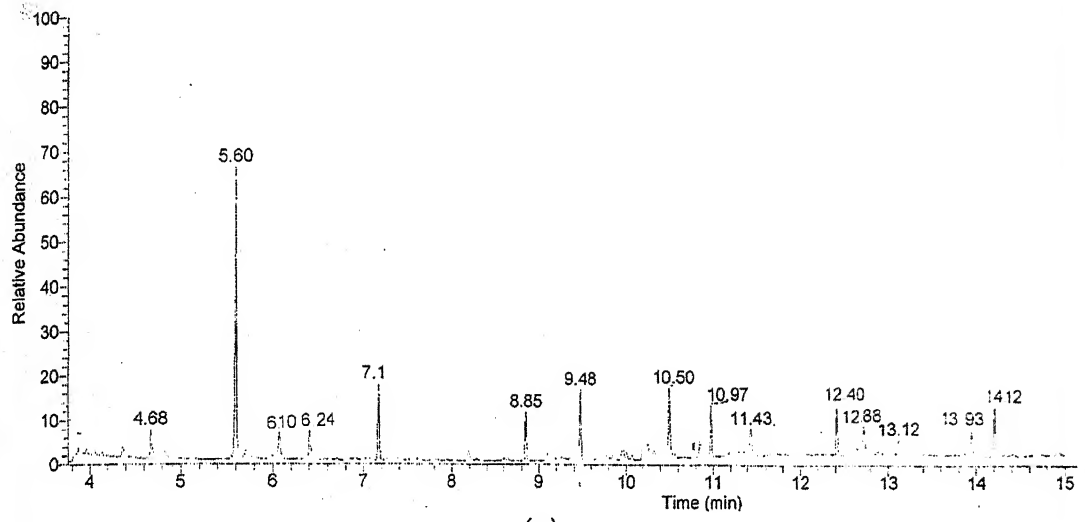
In present study we investigated the effect of UV-B stress on the fatty acid profile of *S. platensis.* This is a first attempt of GC-MS analysis of fatty acids in *S. platensis* under UV-B stress.

Before GC analysis it is necessary to prepare non-reactive derivatives of fatty acids. We used bis-trimethylsilyl trifluoroacetamide (BSTFA) for the silylation of fatty acids. Trimethylsilyl (TMS) ester derivatives are used more widely than any other for the gas chromatographic analysis of hydroxy compounds. Their main value increases the volatility and reduces the polarity of the parent molecules, ensuring sharp symmetrical peaks on GC analysis (Jeong and Lachance, 2001).

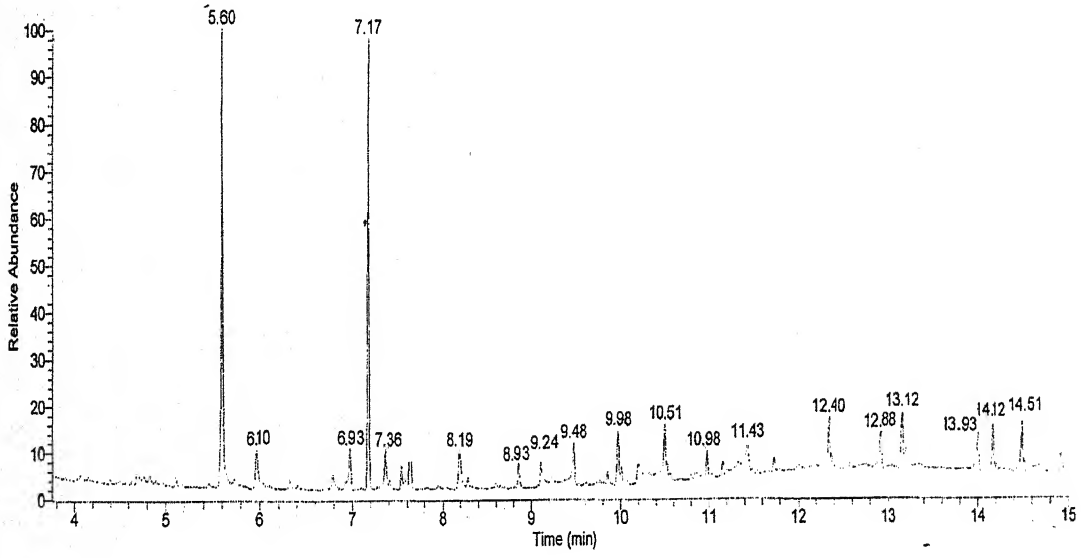
Results of GC-MS analysis of silylated fatty acids in UV-B untreated *S. platensis* shows both saturated and unsaturated fatty

acids. Major constituents of fatty acids were short chain and medium short chain fatty acids (Fig.3.26 a, b). The solvent delay was 4 min. Therefore total ion chromatogram (TIC) is from 4 min (Fig. 3.26a). The saturated fatty acids were octanoic (C_8), nonanoic (C_9), decanoic (C_{10}), dodecanoic (C_{12}), tetradecanoic (C_{14}), hexadecanoic (C_{16}) and octadecanoic (C_{18}). Monounsaturated fatty acids were tetradecenoic ($C_{14:1}$), hexadecenoic ($C_{16:1}$) and polyunsaturated fatty acids were nonadienoic ($C_{9:2}$), decadienoic ($C_{10:2}$), dodecatrienoic ($C_{12:3}$), tetradecatriaenoic ($C_{14:3}$), hexadecatrienoic ($C_{16:3}$) and octadectrienoic ($C_{18:3}$).

Further in contrast to UV-B untreated, GC-MS analysis of silylated fatty acids in UV-B treated *S. platensis* shows short chain and medium short chain saturated, monounsaturated and polyunsaturated fatty acids (Fig. 3.26 b). The dominant saturated fatty acids were decanoic (C_{10}), tetradecanoic (C_{14}), hexadecanoic (C_{16}) and octadecanoic (C_{18}) and in unsaturated fatty acids series both monounsaturated and polyunsaturated fatty acids were obtained. Monounsaturated fatty acids were decaenoic ($C_{10:1}$)



(a)

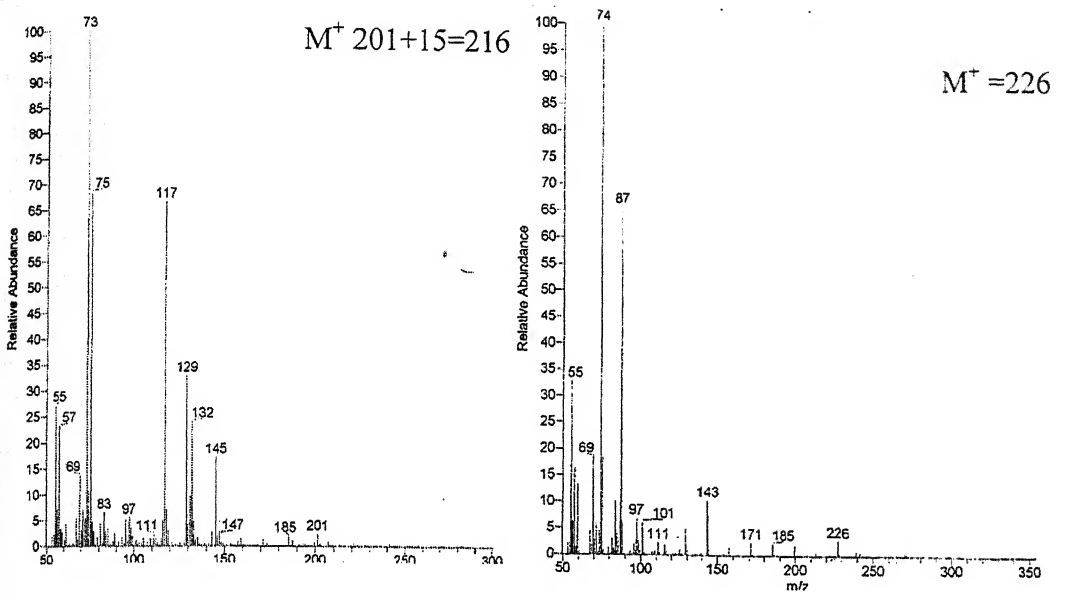


(b)

Fig. 3.26 GC-MS study of trimethylsilyl derivative of fatty acids of UV-B untreated (a) and UV-B treated (b) *Spirulina platensis*

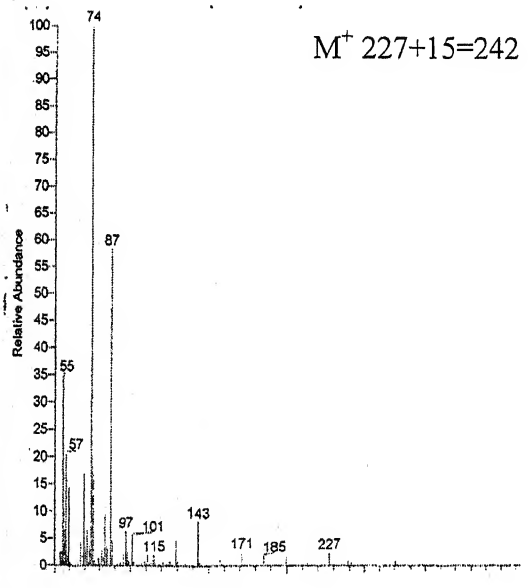
dodecenoic ($C_{12:1}$), tetradecenoic ($C_{14:1}$), hexadecenoic ($C_{16:1}$) and octadecenoic ($C_{18:1}$) and polyunsaturated fatty acids were nonadienoic ($C_{9:2}$), decadienoic ($C_{10:2}$), decatrienoic ($C_{10:3}$), dodecatrienoic ($C_{12:3}$), tetradecatrienoic ($C_{14:3}$), hexadecadienoic ($C_{16:2}$), octadecadienoic ($C_{18:2}$) and octadecatrienoic ($C_{18:3}$) obtained during fatty acids separation.

MS profiles of TMS derivative of fatty acids are shown in Fig. 3.27.1 to 3.27.5. The mass spectra of TMS derivative of fatty acids octanoic, nonadienoic, decaenoic and nonanoic are shown in Fig. 3.27.1. The mass spectra of octanoic (M^+= 216) and decaenoic (M^+= 242) showed $[M-15]^+$ fragment ion at m/z value 201 and 227 respectively which is well known fragmentation of TMS ester of fatty acid (Fig. 3.27.1a, c). The mass spectra of TMS derivative of fatty acids decanoic, dodecatrienoic, dodecenoic and dodecanoic are depicted in Fig. 3.27.2. The mass specta of TMS derivative of fatty acids tetradecanoic, tetradecatrienoic, tetradecenoic and hexadecatrienoic are shown in Fig. 3.27.3. The mass spectrum of tetradecanoic (M^+= 300) showed $[M-15]^+$ fragment ion at m/z 285 which is well known fragmentation of TMS ester of fatty acid

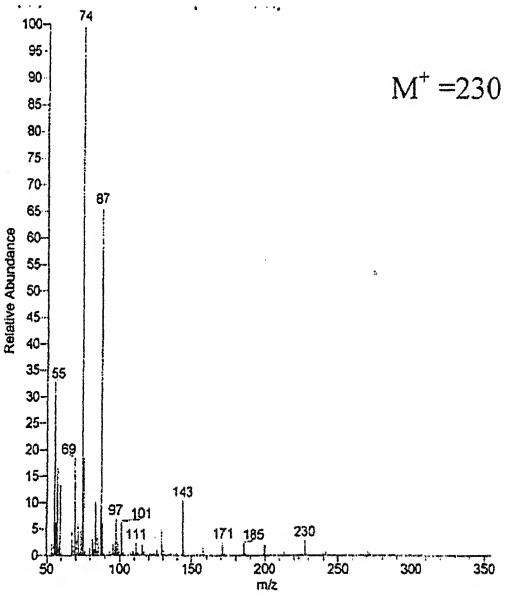


a)

b)



c)



d)

Fig. 3.27.1 Mass spectra of identified peaks of fatty acids of *Spirulina platensis* (C_8 to $C_{10:1}$) a) $C_{8:0}$ b) $C_{9:2}$ c) $C_{10:1}$ d) $C_{9:0}$

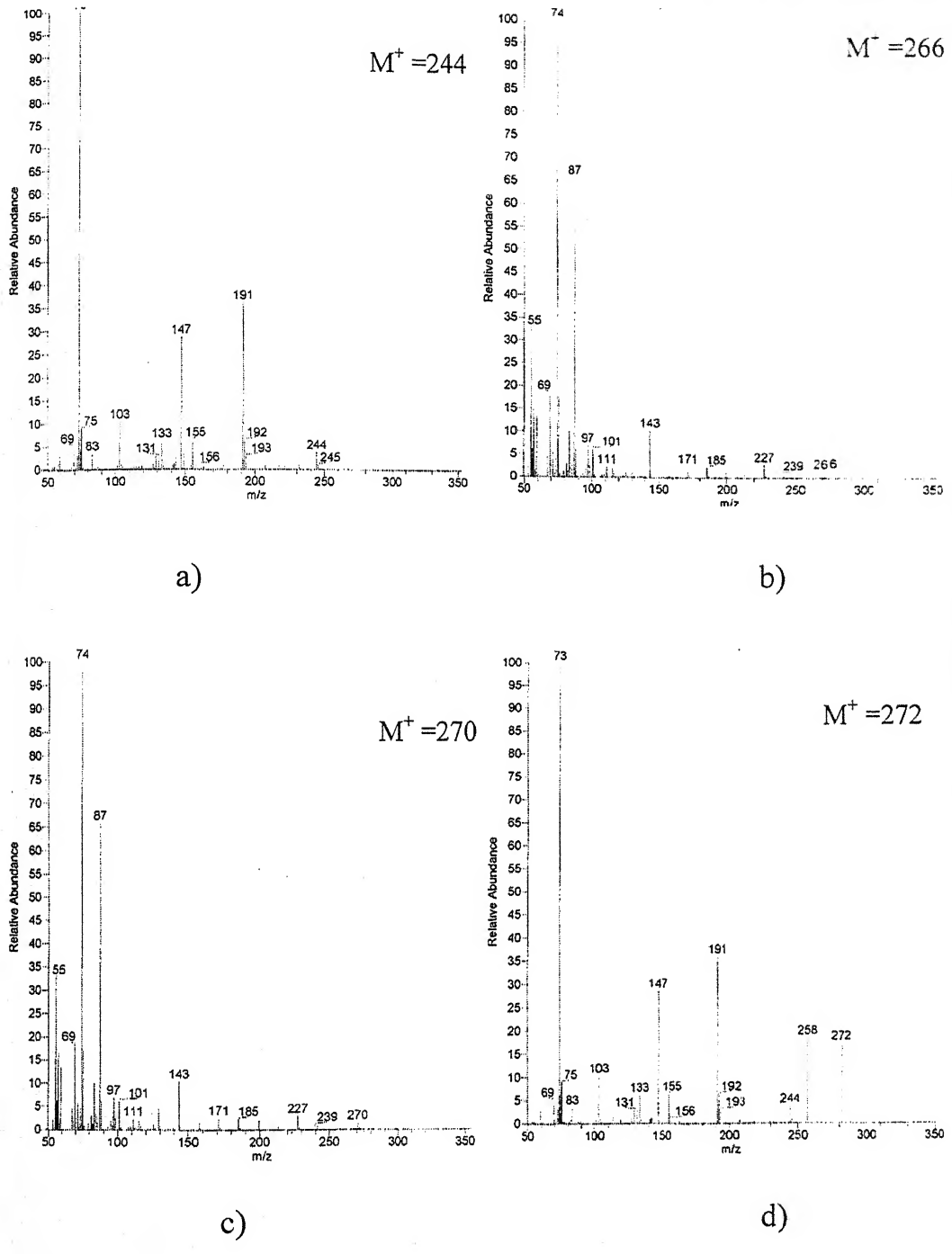
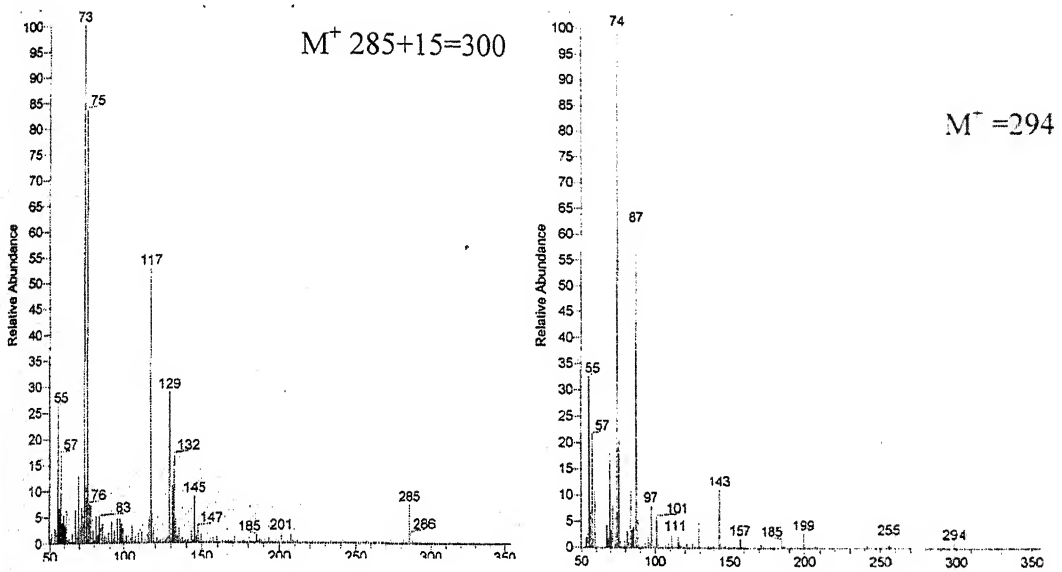
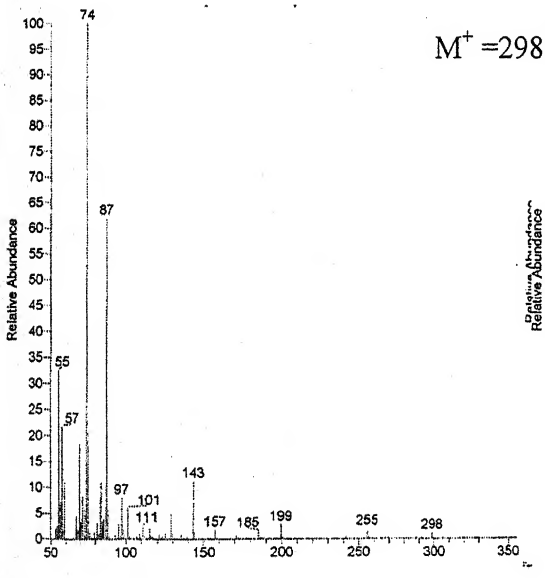


Fig. 3.27.2 Mass spectra of identified peaks of fatty acids of *Spirulina platensis* (C_{10} to C_{12}) a) $C_{10:0}$ b) $C_{12:3}$ c)$C_{12:1}$ d) $C_{12:0}$

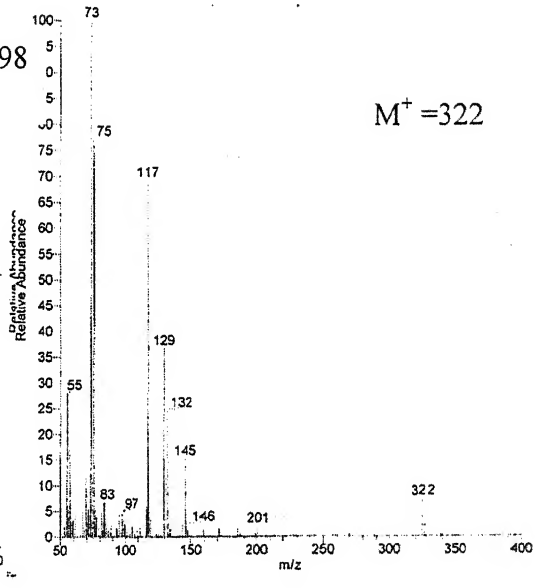


a)

b)



c)



d)

Fig. 3.27.3 Mass spectra of identified peaks of fatty acids of *Spirulina platensis* ($C_{14:3}$ to $C_{16:3}$) a)$C_{14:0}$ b)$C_{14:3}$ c)$C_{14:1}$ d)$C_{16:3}$

(Fig. 3.27.3a). The mass spectra of TMS derivative of fatty acids hexadecadienoic, hexadecenoic, hexadecanoic and octadecatrienoic/ gamma linolenic acid are shown in Fig. 3.27.4. The mass spectra of TMS derivative of fatty acids octadecadienoic, octadecenoic, octadecanoic and decadienoic are shown in Fig. 3.27.5 and mass spectra of decatrienoic are shown in Fig. 3.27.6.

These results indicate that membrane lipid unsaturation increases the tolerance of cyanobacterium to UV radiation. It is evident from the GC-MS result of silylated fatty acid is that the percentage of SFA in UV-B untreated *S. platensis* is 46.7% and the percentage of MUFA and PUFA is 53.3% of total fatty acid content. The percentage of SFA in UV-B treated *S. platensis* is 23.6% and the percentage of MUFA and PUFA is 76.4% of total fatty acid content. Thus UV-B radiation reduced the degree of saturation in *S. platensis* and increased 23.1% unsaturated fatty acid content in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. It is evident from result that in UV-B treated *S. platensis* gamma linolenic ($C_{18:3}$) acid is an important

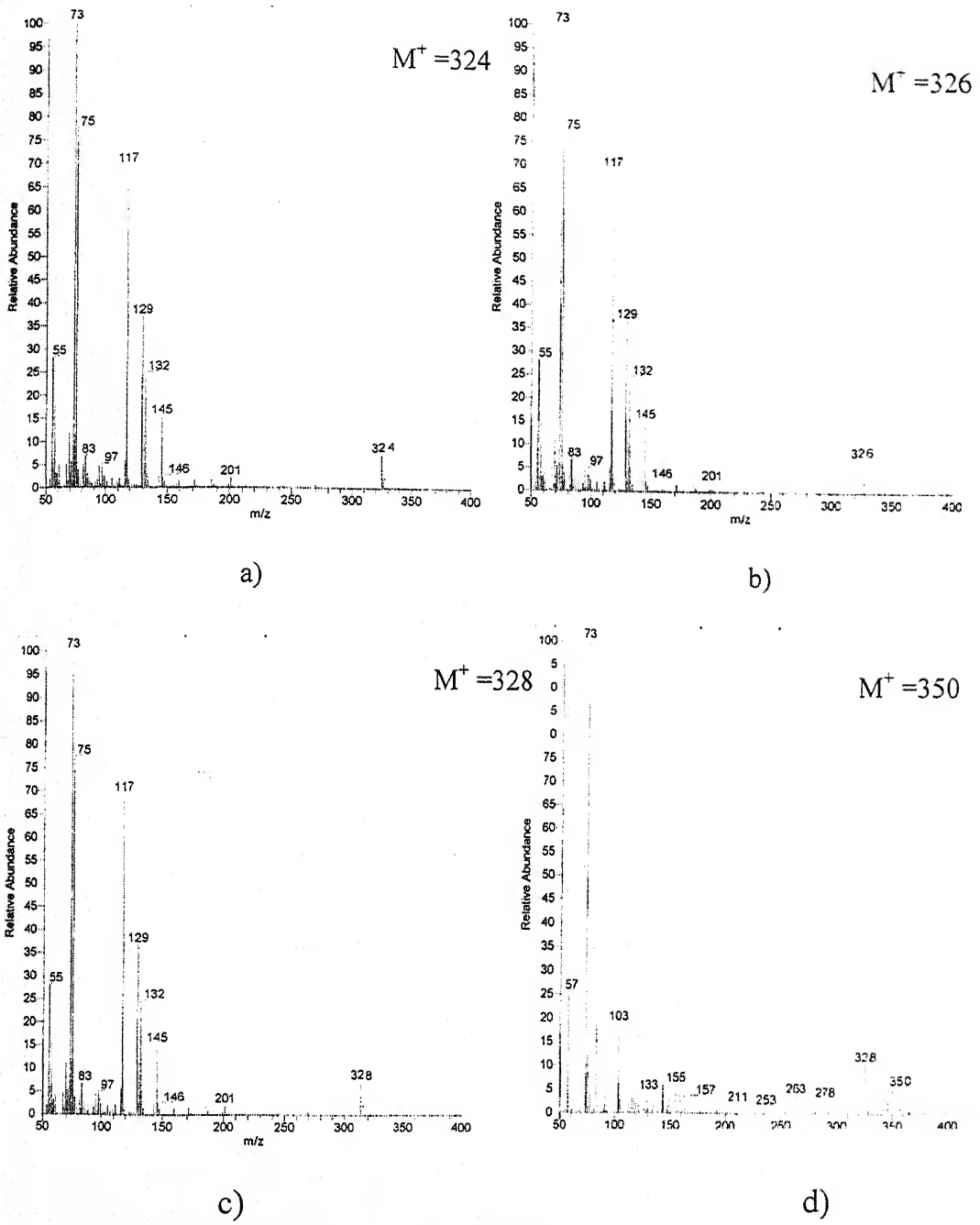


Fig. 3.27.4 Mass spectra of identified peaks of fatty acids of *Spirulina platensis* ($C_{16:2}$ to $C_{18:3}$) a) $C_{16:2}$ b)$C_{16:1}$ c)$C_{16:0}$ d)$C_{18:3}$

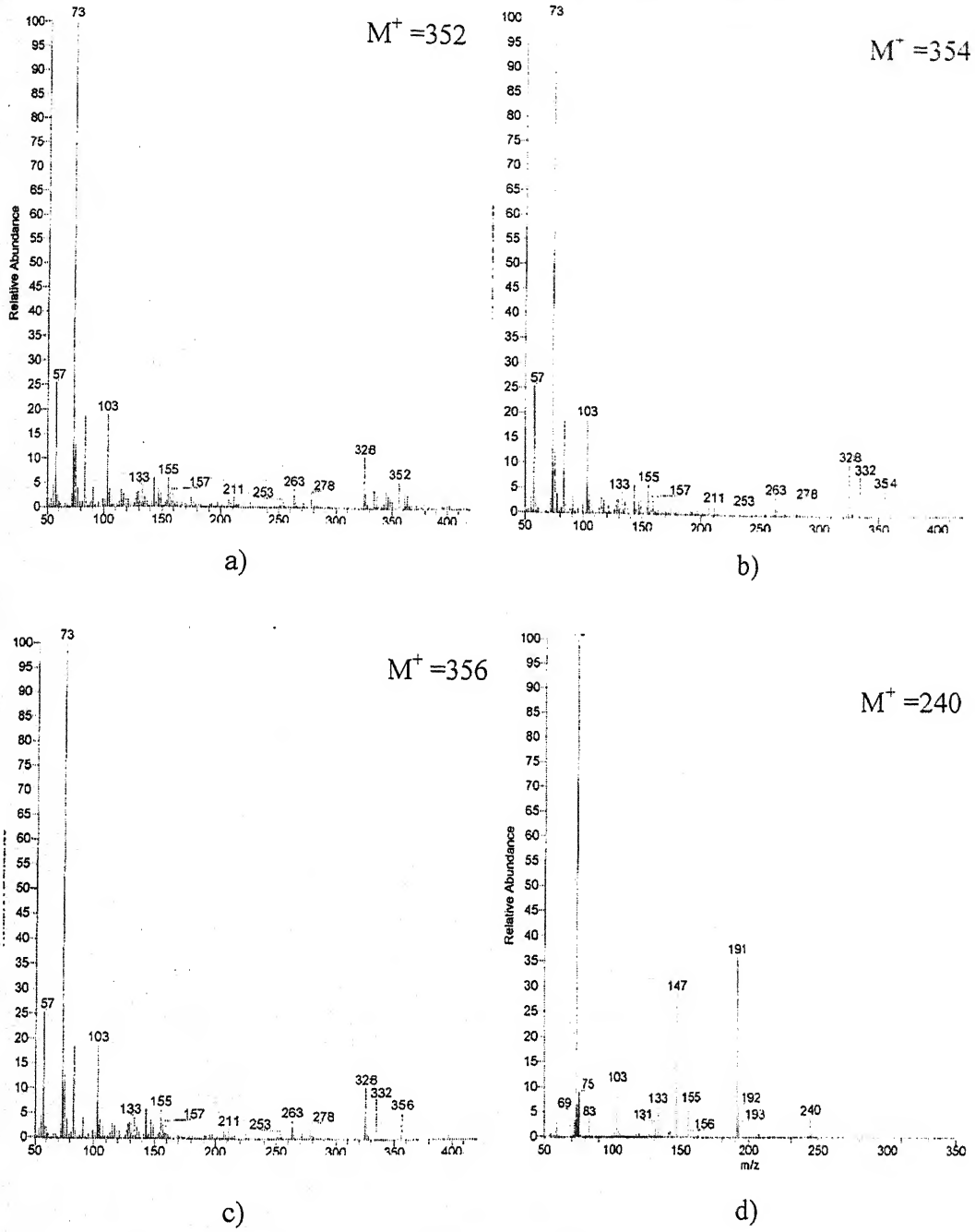


Fig. 3.27.5 Mass spectra of identified peaks of fatty acids of *Spirulina. platensis*, a) $C_{18:2}$ and b) $C_{18:1}$ c)$C_{18:0}$ d)$C_{10:2}$

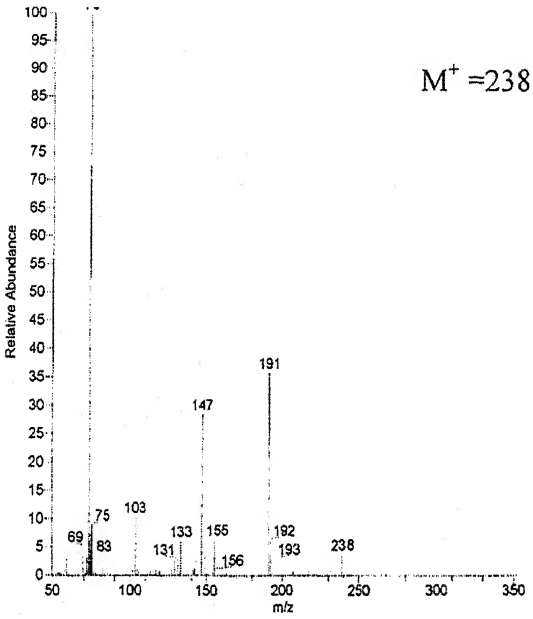


Fig. 3.27.6 Mass spectra of identified peaks of $C_{10:3}$ fatty acids of *Spirulina. platensis*

component of total content of PUFAs as compared to UV-B untreated counterpart.

This result indicates that in tolerance of UV-B radiation membrane lipid unsaturation play important roles in cyanobacterium *S. platensis*. Previous studies showed that unsaturation of fatty acids are important in regulating membrane fluidity and physiological processes under stress (Hall *et al.,* 2002, Gombos *et al.,* 1997; Hessen *et al.,* 1997).

3.6 Effect of UV-B radiation on Hydrcarbon profile of *S. platensis*

Most cyanobacteria are a common source of a wide range of fatty acids, hydrocarbons and sterols with potential not only as a renewable source of liquid fuels but also for the production of a range of pharmacologically and industrially important products. Hydrocarbons are also used as sole carbon and energy source (Walker and Pore, 1978).

S. platensis is good source of hydrocarbons (Tulliez *et al.,* 1975; Fedelio and Favini, 1980), having antimicrobial activity against wide spectrum for four Gram-positive, six Gram-negative

bacteria and *Candida albicans* ATCC 10239 and anti HIV activity (Burja *et al.,* 2001; Ozademir *et al.,* 2004).

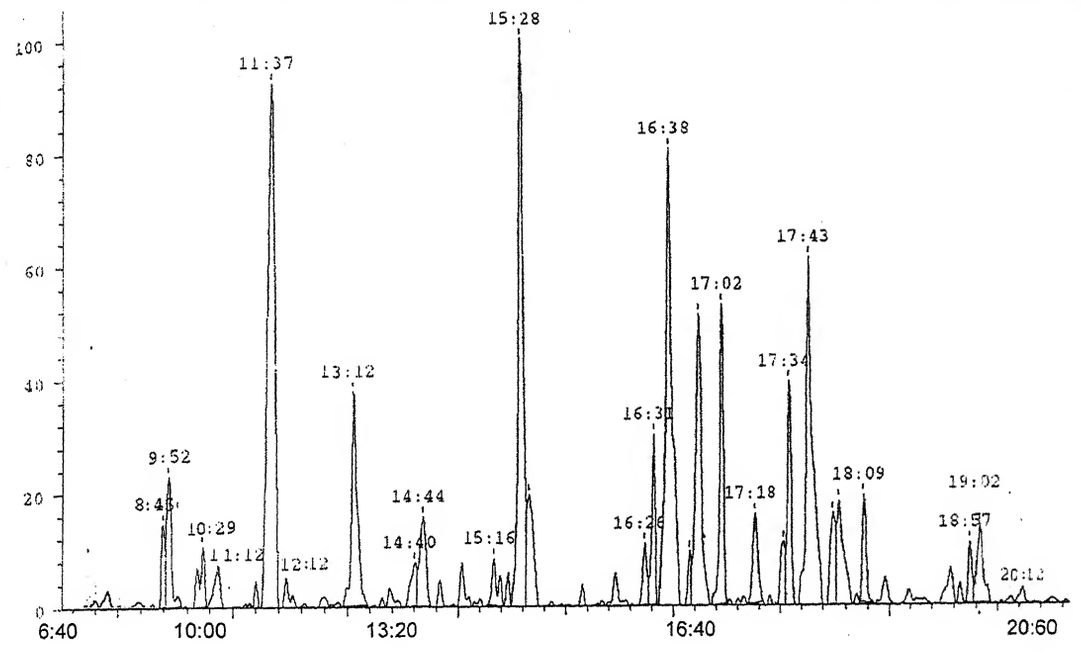
The Long-chain saturated hydrocarbons represent a substantial fraction 25% of the non-saponifiable element in *S. platensis* (Bujard *et al.*, 1970). Thus, dry *S. platensis* contains between 0.1% and 0.3% of saturated hydrocarbons. Two-thirds of these hydrocarbons consist of n-heptadecane, the remainder, in descending order, of saturated linear hydrocarbons (C_{15}, C_{16}, C_{18}) and three unidentified saturated branched-chain hydrocarbons (Tulliez *et al.*, 1975).

In present study we investigated the n-alkanes profiles specially heptadecane and tetradecane in UV-B untreated and UV-B treated *S. platensis* through GC-MS. The cells of *S. platensis* were harvested and extracted with hexane and the unsaponifiable fraction was used for hydrocarbon analysis. The n-alkanes were separated by serially coupled capillary column to mass detector.

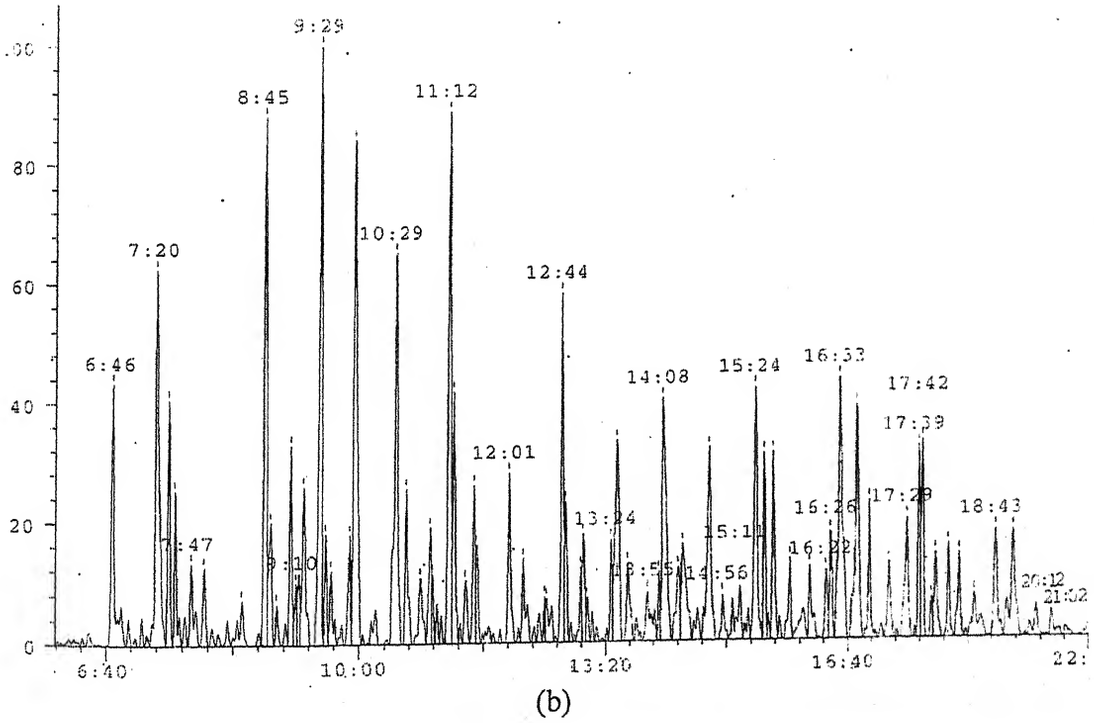
GC-MS analysis of hydrocarbons in UV-B untreated *S. platensis* indicated both short and long chain n-alkanes

(Fig.3.28 a, b). The solvent delay was 6 min. Therefore, complete total ion chromatogram (TIC) is from 6 min (Fig. 3.28). The major constituents of short chain n-alkanes are nonane (C_9H_{20}), tetradecane ($C_{14}H_{30}$), hexadecane ($C_{16}H_{34}$), heptadecane ($C_{17}H_{36}$) and octadecane ($C_{18}H_{38}$) and n-cosane ($C_{2O}H_{42}$), and monocosane ($C_{21}H_{44}$), docosane ($C_{22}H_{46}$), triecosane ($C_{23}H_{48}$), tetracosane ($C_{24}H_{50}$) and hexaacosane ($C_{26}H_{54}$) were the major constituents of long chain n-alkane in UV-B untreated *S. platensis*. The major constituents of short chain n-alkane were decane ($C_{10}H_{22}$), tetradecane ($C_{14}H_{30}$), pentadecane ($C_{15}H_{32}$), heptadecane ($C_{17}H_{36}$), octadecane ($C_{18}H_{38}$) and cosane ($C_{2O}H_{42}$), and long chain n-alkanes were monocosane ($C_{21}H_{44}$), docosane ($C_{22}H_{46}$), tetracosane ($C_{24}H_{50}$), pentacosane ($C_{25}H_{52}$), hexacosane ($C_{26}H_{54}$), heptacosane ($C_{27}H_{56}$), octacosane ($C_{28}H_{58}$), triacontane ($C_{30}H_{62}$) and tetratriacontane ($C_{34}H_{70}$).

It is evident from the GC-MS result on hydrocarbon that the percentage of short chain n-alkanes (C_9-C_{20}) is 54.6% and long chain alkanes (C_{21}-C_{34}) is 45.4% of total hydrocarbon content in UV-B untreated *S. platensis*. The percentage of short chain



(a)



(b)

Fig. 3.28 GC-MS study of hydrocarbons of UV-B untreated (a) and UV–B treated (b) *Spirulina platensis*

n-alkanes is 40% and long chain n-alkanes is 60% of total hydrocarbon content in UV-B treated *S. platensis.* Thus, the GC-MS results show that 14.6% long chain alkanes (C_{21}-C_{34}) increased of total alkanes in UV-B treated *S. platensis* as compared to UV-B untreated counterpart (Fig.3.28 a, b).

MS spectrum reveals that the m/z values 199; $[M-1]^+$ for tetradecane ($C_{14}H_{30}$) and 241; $[M-1]^+$ for heptadecane ($C_{17}H_{36}$) are shown in Fig. 3.29a,b. The part of TIC for tetradecane (C_{14}) and heptadecane (C_{17}) of UV-B untreated and UV-B treated *S. platensis* is depicted in Fig.3.30a, b. Result shows that the level of tetradecane (C_{14}) and heptadecane (C_{17}) were increased in UV-B treated *S. platensis* as compared to UV-B untreated counterpart (Fig.3.30a, b).

Both hydrocarbon tetradecane (C_{14}) and heptadecane (C_{17}) has potent antimicrobial activity (Ozademir *et al.*, 2004). But further investigation is needed to understand the mode of action and also for study of antimicrobial activity of tetradecane (C_{14}) and heptadecane (C_{17}).

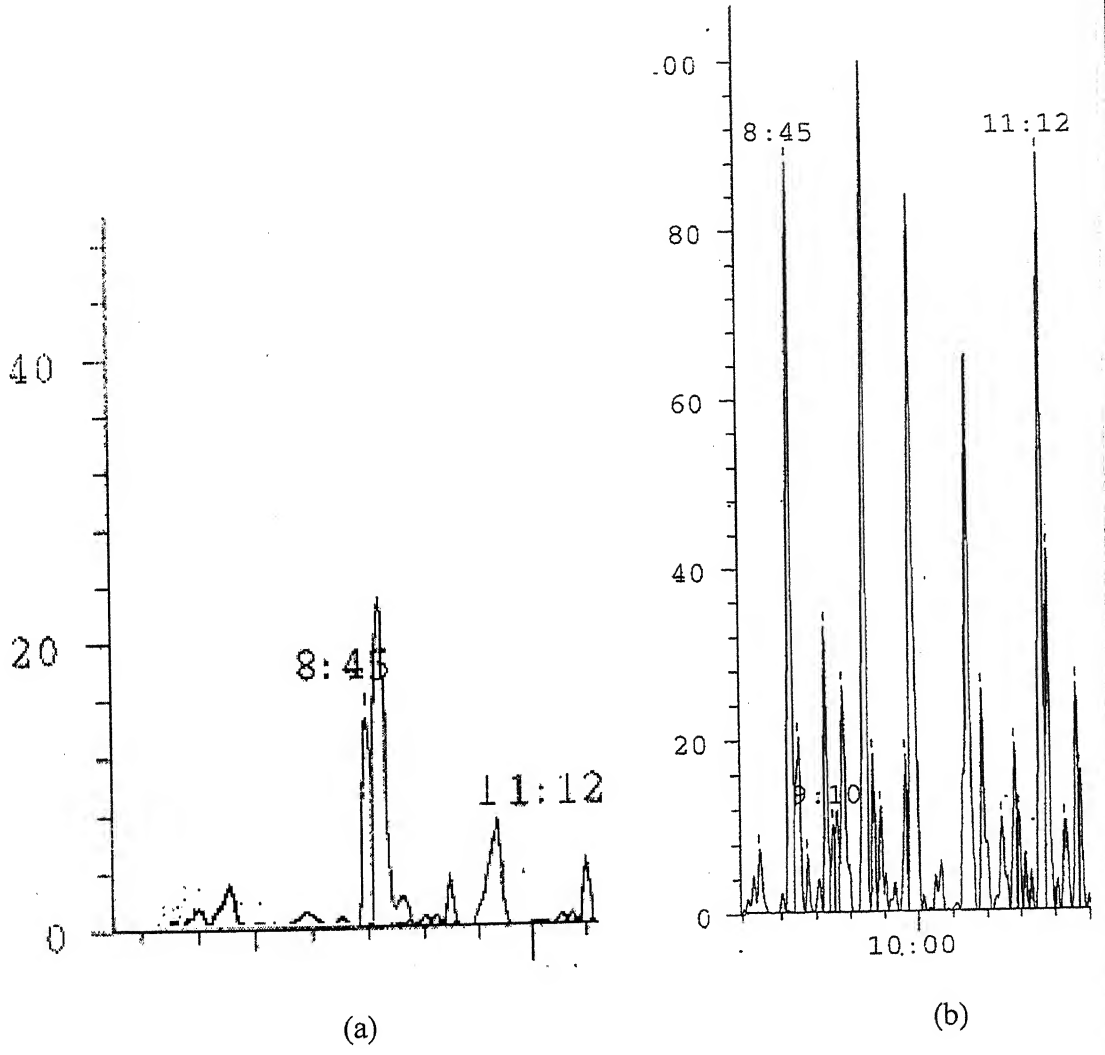
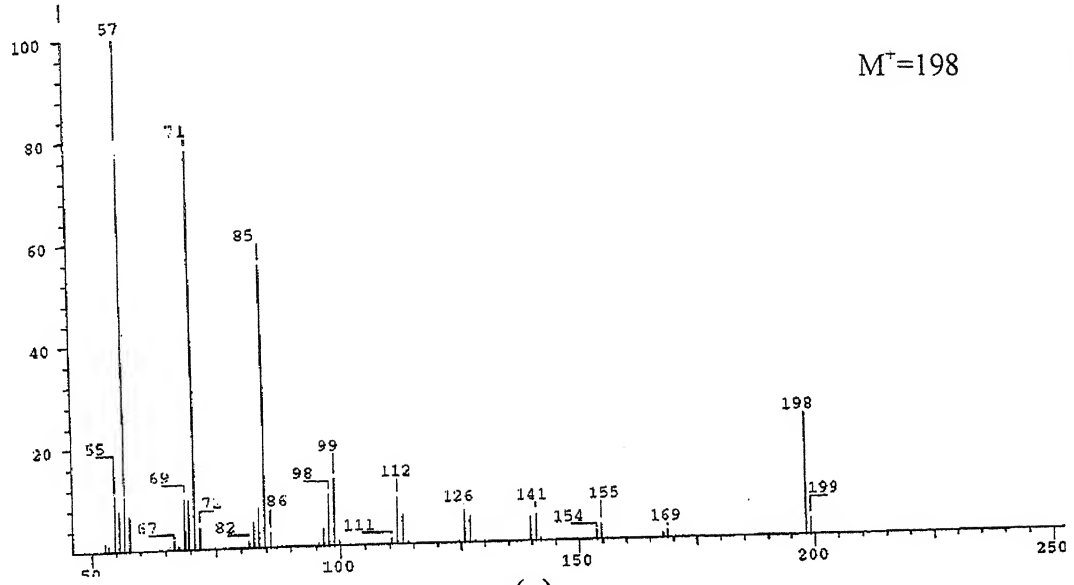
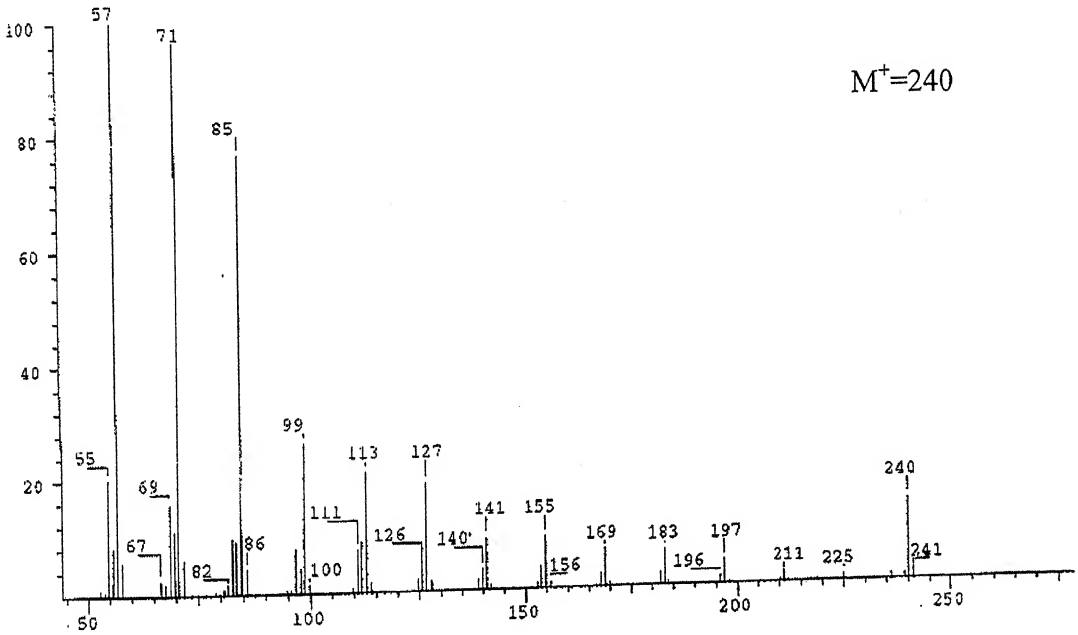


Fig. 3.29 Part of GC-MS total ion chromatogram of hydrocarbons n-tetra decane and n-hepta decane of UV-B untreated (a) and UV-B treated (b)*Spirulina platensis*



(a)



(b)

Fig. 3.30 EI-MS of identified peak of hydrocarbon n-tetra decane (a) and n-hepta decane (b) of *Spirulina platensis*

Result shows that under UV-B stress the n-alkane content in *S. platensis* were increased as compared to UV-B untreated counterpart. The n-alkane content increased in UV-B treated *S. platensis* for protecting the cell from environmental stress. Similar results have been found in leaves of higher plants containing waxy alkanes, which are useful for protection against UV-B radiations, as well as photoinhibition (Robinson *et al.*, 1993).

Dembitsky and Srebnik, (2002) studied the dominant components of hydrocarbons in culture of filamentous cyanobacterium *Scytonema* sp. and isolated similar long chain hydrocarbons, n-alkane that ranged from C_{12} to C_{25} in lake water samples (Badawy *et al.,* 1999), n-alkane in lichen (Zygadio *et al.,* 1993), in photobionts a green algae *Trebouxia sp* (Toress, 2003) and hydrocarbons and volatile components in cyanobacterium Nostoc sp. (Dembitsky *et al.,* 1999).

Biologically active compounds like n-alkanes isolated from *S. platensis* have an antimicrobial activity (Ozademir, 2004) and are also rich source of nutritional and therapeutic potential.

General Discussion

Spirulina is a filamentous, photosynthetic, multicellular cyanobacterium belonging to Oscillatoriaceae family, characterized by spiral shaped and enclosed in a thin sheath with a long history of being use as food supplement (Ciferri, 1983; Belay *et al.,* 1994, 1996, 1997; Blinkova *et al.,* 2001). In the present study, we used UV-B radiation to improve the potential of *Spirulina* with reference to it's contents like carbohydrate, fatty acid and hydrocarbons and so on.

The depletion of the stratospheric ozone layer, mainly due to anthropogenically released pollutants such as chlorofluorocarbons, has resulted in an increase in the solar UV-B radiation (280 to 315 nm) that reaches the Earth's surface (Crutzen, 1992; Kerr and McElroy, 1993). Ozone depletion is expected to increase and to spread over a broader range of altitudes and latitudes throughout in the current century (Tabazadeh *et al.,* 2000). UV-B radiation is potentially detrimental to all forms of life but is more detrimental to photosynthetic organisms, including cyanobacteria (Häder, 2000; Sinha *et al.,* 1996; Stapleton, 1992).

As we know, on the basis of utility, *Spirulina* can be cultured under variable natural, artificial and laboratory conditions. Nutrient content of *Spirulina* depends on the location and environment in which the cyanobacterium grows. Percentage of specific components of *Spirulina* can be increased or decreased according to need by growing under regulated growth conditions. Zarrouk's modified medium having some trace element for the growth was found to be better for the growth of *Spirulina* as compare to Zarrouk's basal medium.

The growth rate (in terms of protein, chl *a*, dry weight and specific growth rate) of *Spirulina* was higher on ZM medium as compared to ZB. 21 kD protein was expressed only in Zarrouk's modified medium and the expressions of other proteins were similar on both culture medium. The synthesis of the new proteins in response to culture medium plays an important role in the maintenance of vital cellular functions in cyanobacteria.

UV-B radiation affected the morphology of *Spirulina*. It is confirmed by various microscopical examinations (bright field, fluorescent and SEM microscopy) that UV-B radiation affected the

morphology of cyanobacteria due to adaptation and protections of cells from UV stress. It was further observed that UV-B radiation (280-320 nm) not only affects the growth rate (in reference to protein and chl *a*) but also enhances the potential of *Spirulina* to grow under unfavorable conditions. Cells are also protected from stress by secretion of osmoregulatory compound.

The synthesis of carbohydrate content in UV-B untreated *S. platensis* was 21.97%, and for UV-B treated *Spirulina* was 34%. There was an increase in carbohydrate content by 12% in UV-B treated *Spirulina* as compared to UV-B untreated counterpart. UV-B radiations stimulate biosynthesis of carbohydrate which meets the additional requirement of energy for protecting the cell from stress.

The rate of nitrate uptake and nitrite uptake in UV-B treated *Spirulina* was lower as compared to UV-B untreated counterpart. It has been reported that UV-B exposure has a deleterious effect on the photosynthetic apparatus leading to the reduction in the supply of ATP and $NADPH_2$ (Kulandaivelu and Noorudeen, 1983). As such, disruption of cell membrane and /or alteration in thylakoid

integrity as a result of UV-B radiation may partly damage the photosynthetic apparatus (Vu *et al.,* 1981). Thus, there is a possibility that the inhibition in NR and NiR activity might be due to the reduced supply of reductants and energy following the UV-B treatment.

The present study also deals with the study of the changes at the level of thylakoid membrane. Thylakoid membranes of *S. platensis* showed high chl *a* content and thickening on the outer side of the membrane as compared to UV-B treated counterpart which may be due to high hill activity and CO_2 fixation in UV-B untreated organism. Results suggested that under UV-B stress, the outer membrane of *Spirulina* become partially distorted. Two proteins of 20 and 94 kD were exclusively synthesized in the thylakoid membrane of UV-B treated *S. platensis* suggesting that they might be playing a key role as specific/ selective carrier under UV stress in *Sprirulina* (Rajgopal *et al.,* 1998, 2000). LCMS result also shows that level of chl *a* is decreased in the thylakoid membrane of UV-B treated *Spirulina* as compared to UV-B untreated counterpart. High-pressure liquid chromatography and field mass spectrometry were studied in order to analyse pigments

under stress (Evans, 1975). Our study clearly reveals that prolonged UV-B exposure drastically *alters* the absorption and fluorescence emission spectrum of the thylakoid membranes. Rajgopal *et al.,* (1998, 2000) suggested that prolonged UV-B irradiation affects the chl a-protein complexes of the thylakoid membranes in cyanobacteria.

Our investigations further deal with the effect of UV-B radiation on fatty acid profile of *Spirulina*. As per GC-MS result, the percentage of SFA (silylated fatty acid) is 46.7% and the percentage of MUFA and PUFA is 53.3% of total fatty acid content in UV-B untreated *S. platensis*. The percentage of SFA is 23.6% and the percentage of MUFA and PUFA is 76.4% of total fatty acid content in UV-B treated *S. platensis*. UV-B radiation reduced the degree of saturation in *Spirulina* and increased 23.1% unsaturated fatty acid content in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. The membrane lipid unsaturation is increased due to the tolerance of cyanobacterium to UV radiation (Gombos *et al.,* 1997). Similar conclusions have been drawn in other phytoplankton cells (Hessen *et al.,* 1997).

GC-MS result on hydrocarbon shows that the percentage of short chain n-alkanes (C_9-C_{20}) is 55.6% and the percentage of long chain n- alkanes (C_{21}-C_{34}) is 44.4% of total hydrocarbon content in UV-B untreated *S. platensis*. The percentage of short chain n-alkanes (C_9-C_{20}) is 40% and the percentage of long chain n-alkanes (C_{21}-C_{34}) is 60% of total hydrocarbon content in UV-B treated *S. platensis.* Thus UV-B radiation increased 14.6% long chain n-alkanes (C_{21}-C_{34}) content of total hydrocarbon content in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. Result further suggested that under UV-B stress the n-alkane content in *S. platensis* were increased as compared to UV-B untreated. The level of tetradecane and heptadecane were also increased in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. Ozademir *et al.,* (2004) suggested that tetradecane (C_{14}) and heptadecane (C_{17}) has potent antimicrobial activity.

The n-alkane content increased in UV-B treated *S. platensis* for the protection of cell from environmental stress. Similar results have been reported in leave of higher plants containing waxy

alkanes, which are useful for protection against UV-B radiations and photo inhibition (Robinson, 1993).

Thus, overall it can be concluded that UV-B radiation affects the synthesis of polyunsaturated fatty acids (PUFAs), carbohydrate and n-alkanes in *S. platensis* significantly. But protein and chlorophyll content was decreased as a result of UV-B radiation. *Spirulina* would, therefore, be an excellent source of PUFAs, carbohydrate and n-alkane for the reclamation solving the problem of malnutrition and fuels.

Summary

The genus *Spirulina*, is the most important commercially cultivated cyanobacterium, due to its high nutritional value, chemical composition and safety of its biomass for human consumption. It is cultivated on a large scale as a monoculture in intensive outdoor cultivation systems.

The present study was aimed to enhance the potential by UV-B radiation for the production of *Spirulina* as a cheap alternate source of polyunsaturated fatty acids (PUFAs), carbohydrate and hydrocarbon for nutritions and fuels. UV-B treatments make cyanobacterial strain resistant to unfavorable conditions and protect it by secretion of osmoregulatory compound.

UV-B treated *Spirulina* unlike to its UV-B untreated counterpart showed morphological variations in terms of granulation, pigmentation and both apical and terminal end of filaments. And the thylakoid membrane of UV-B treated and UV-B untreated were isolated by mechanical disruption of the freeze dried and lysozyme-treated cells followed by differential and density gradient centrifugation. Thylakoid membranes of *S. platensis* showed morphological variation in high chl *a* content and thickening on the outer side of the membrane as compared

to UV-B treated counterpart. The absorption and fluorescence spectra of chl *a* of the thylakoid membrane is altered by UV-B radiation. LC-MS analysis of pigment; chl *a* of thylakoid membrane of *Spirulina* also revealed that UV-B radiation reduced the chl *a* level in UV-B treated *Spirulina* as compared to UV-B untreated counterpart. This suggests that changes in membrane morphology under UV-B stress is due to the induction of stress proteins, which keep thylakoid membrane alive under UV-B stress.

UV-light exposure can cause reactive oxygen species generation (ROS) such as O_2- and H_2O_2 (Shibata *et al.,* 1991). Though, H_2O_2 is an innocuous metabolite present in cells irradiation with UV-light breaks it down to extremely deleterious hydroxyl free radicals (OH^-). Since, H_2O_2 can easily diffuse through cell membranes it is extremely deleterious to cellular constituents such as DNA and other biological compounds. Several studies have indicated that *in vitro* anthocyanins could act as effective antioxidants (Sarma *et al.,* 1997). UV-B radiation leads to photoinhibition of photosynthesis thereby limiting the efficient fixation of light energy (Han *et al.* 2001, Nishiyama *et al.* 2001).

Photoinhibition occurs due to two basic mechanisms: (i) photoinduced, nonphotochemical quenching of excitation energy and (ii) photoinduced damage to the photosynthetic machinery (Krause 1988). In cyanobacterial photosynthesis, the nonphotochemical quenching particularly measured by O_2 evolution is not induced by light, indicating that the photoinhibition is mainly due to the photoinduced damage to the photosynthetic machinery. The molecular mechanism of photoinhibition, further, revealed that the light-induced damage is caused by inactivation of the D1 protein of the PSII complex (Aro *et al.* 1993, Kanervo *et al.* 1993; Tyystjärvi *et al.* 2001). The damaged D1 protein is degraded proteolytically leaving the PSII complex depleted of the D1 protein. In the recovery process the precursor of the D1 protein is synthesized *de novo*, incorporated into the PSII complex, and then processed to yield the active D1 protein, with resultant generation of the active PSII complex (Andersson *et al.* 1992). The extent of the photoinhibition depends on the balance between the inactivation of the PSII complex and the recovery of the complex from the inactivated state (Gombos *et al.,* 994).

GC-MS analysis of fatty acid profile showed that UV-B radiation enhance the PUFAs of UV-B treated *Spirulina* as compared to low levels of PUFAs in UV-B untreated *Spirulina,* suggesting that membrane lipid unsaturation is increased for the tolerance of cyanobacterium to UV radiation, which make cell alive in stress.

GC-MS analysis of hydrocarbon showed that UV-B radiation enhance especially long chain n-alkanes (C_{21}-C_{34}) in UV-B treated *Spirulina* as compared to low levels of long chain n-alkanes in UV-B untreated *Spirulina,* suggesting that hydrocarbon. is also increased for the tolerance of cyanobacterium to UV radiation, which make cell alive in stress.

Thus overall it can be concluded that effect of UV-B radiation increased contents of polyunsaturated fatty acids (PUFAs), carbohydrate and n-alkanes in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. But protein and chlorophyll content was decreased in UV-B treated *S. platensis* as compared to UV-B untreated counterpart. UV-B treated *Spirulina* would be a better source of PUFAs, carbohydrate and n-alkane for the reclamation solving the problem of malnutrition and fuels.

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